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Nucleobindin-2: A Novel Regulator of Immune-Metabolic Interactions

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NUCLEOBINDIN-2: A NOVEL REGULATOR OF IMMUNE-METABOLIC INTERACTIONS

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List of Abbreviations

ADP- Adenosine 5’-diphosphate
ATM- Adipose Tissue macrophages
ATP- Adenosine 5’-triphosphate
BMDM- Bone Marrow Derived Macrophages
CR- Caloric Restriction
DAMP- Danger Associated Molecular Pattern
DIO- Diet Induced Obesity
EGP- Endogenous Hepatic Glucose Production
ETC- Electron Transport Chain
FFA- Free Fatty Acids
GLUT- Glucose Transporter
HFD- High Fat Diet
I.C.V. - Intracerebroventricular
IFN- Interferon
IRS- Insulin Receptor Substrate
ISG- Interferon Stimulated Genes
LPS- Lipopolysaccharide
MHC- Major Histocompatibility Complex
NEFA- Nonesterified Fatty Acid
Nucb2- Nucleobindin 2
PAMP- Pathogen Associated Molecular Pattern
PRR- Pattern Recognition Receptor
SAT- Subcutaneous Adipose Tissue
SVF- Stromal Vascular Fraction
TNF- Tumor Necrosis Factor
TLR- Toll-like Receptor
T2D- Type 2 Diabetes
VAT- Visceral Adipose Tissue
WHO- World Health Organization

Abstract

Over the past half century, obesity has become a widespread concern to human health. Obesity is defined as having a body mass index above 30 and is accompanied by altered metabolic physiology. Obesity is one of the leading causes of mortality in the United States and in the world. The prevalence of obesity [1] has increased over the past few decades. High body fat in adipose, excess calorie intake, low aerobic fitness are all associated with the comorbidities of obesity [2, 3] [4-6] [7-13]. Recently, an emphasis has been given to increased leukocytes in adipose tissue and the resulting low-grade systemic inflammation on the development of insulin resistance [4-6]. Unfortunately, the immune-metabolic molecular mechanisms by which increased inflammation leads to diseases is not fully understood.

We studied *Nucb2,* a highly expressed gene in immune cells and encodes for nesfatin-1, a peptide reported to be a satiety signal [14]. We saw that *Nucb2* expression is increased in leukocytes of high fat diet (HFD) fed animals. Contrary to our hypothesis, *Nucb2-/-* animals showed no effect on food intake. We then performed euglycemic hyperinsulinemic clamp studies in wild type and *Nucb2* mice on a chow diet and a HFD to determine insulin sensitivity. Interestingly, knocking out the *Nucb2* gene significantly impaired insulin sensitivity only under HFD conditions. These experiments demonstrate that *Nucb2* is key player in glucose homeostasis in obesity.

We discovered macrophages deficient of *Nucb2* impact the proinflammatory macrophages. Macrophages lacking *Nucb2* increased proinflammatory cytokine production. This suggests that *Nucb2* intrinsically regulates the inflammatory cytokine production cascade.

Mechanistically, *Nucb2* mediates its effects by increasing proinflammatory cytokine expression via down-regulation of NFκB signaling in leukocytes. In the absence of *Nucb2,* NFκB activity is increased in macrophages. In contrast, inhibiting NFκB caused the opposite response. Finally, in response to endotoxemia, *Nucb2* is required to control the production proinflammatory cytokines. Collectively, these data highlight a novel mechanism whereby Nucb2 serves as a key mediator of immunometabolic control of inflammation and insulin resistance. Overall, the studies I have performed identified a novel mediator of the immune-metabolic cross talks in the context of obesity-induced insulin resistance.

Chapter 1: Introduction

1.1 Background and Significance

Obesity is a disease that has recently reached epidemic proportions in developed as well as in developing countries [15], and is now recognized as a more complex condition than once believed. Obesity is a multifactorial condition in which environmental risk factors related to a sedentary life-style and excess access to food apply constant pressure in subjects with a genetic predisposition to gain weight [16]. The role of genetics in obesity is complex, as it involves the interaction of many genes which may each have relatively small effects, yet work in combination with others and with external environmental factors to ultimately cause weight gain. Despite evidence that genes are involved in the variation of body fat in humans, no major obesity gene was identified until the *ob* gene was discovered in mice [17]. However, the absence of leptin (the product of the ob gene) is affecting only a few people in the world. As obesity has become more prevalent throughout the world, researchers have found a strong association between obesity and type 2 diabetes, now also reaching epidemic levels around the world.

Type 2 diabetes is characterized by a decrease in glucose uptake in cells (such as muscle, liver and adipose) resulting, in part, from defects in insulin sensitivity. This decrease in insulin sensitivity is often further affected by defects in insulin secretion. Together, these deleterious consequences of insulin resistance and progressive beta-cell failure to secrete insulin results in increased blood glucose levels, leading to blood vessel damage and eventually to tissue and organ failure [18, 19].

Recently, evidence has been found for a connection between inflammation in adipose tissue and insulin resistance. Adipose tissue is traditionally considered to be a long-term energy storage organ, but now, it is also known to play key roles in the integration of systemic metabolism. Excess body fat leads to systemic inflammation [20]. This inflammatory state is initiated largely in the adipose tissue, which itself releases cytokines, adipokines and fatty acids. Downstream, deleterious effects could ensue on the liver and in the muscle which are key organs and tissues for the regulation of whole body glucose metabolism [6, 20-22].

The immune system includes many complex biological structures and processes within an organism that protects against disease. A properly functioning immune system must detect a wide variety of agents, known as pathogens, from viruses to parasites, and distinguish them as foreign from the organism's own healthy tissues. Another fundamental feature of a properly functioning immune system is the ability to terminate the immune response after the resolution of infection. This is necessary to prevent autoimmunity and to reduce ongoing inflammation.

Unfortunately, the molecular mechanisms or regulatory genes that mediate the immune-metabolic crosstalk that drive obesity-related inflammation and chronic diseases are not fully understood. For example, several satiety regulatory peptides that act on the hypothalamus and act as long-term signals of energy balance, can also activate many immune cells. Ghrelin and leptin, for example, are produced in peripheral sites such as the gut and adipose tissue respectively and play roles in body weight regulation as well as immune cell functions. Dysregulation of these immune functions can therefore contribute to the pathophysiology of insulin resistance and its associated metabolic complications such as diabetes [4-6].

As a consequence, special efforts have recently focused on understanding the molecular mechanisms of "sterile inflammation". The specific mechanisms and regulatory genes that drive metabolic-related inflammation and chronic diseases via mediation of the immune-metabolic crosstalk are not fully comprehended. Emerging genes, such as nucleobindin-2 (*Nucb2*) and Nesfatin-1 are required for maintenance of normal immune-metabolic interactions. As an example, Nesfatin-1, a proteolytically cleaved peptide of *Nucb2*, has been shown to be a potent anorexigenic protein reducing food intake in rodents when administered either centrally or peripherally [14, 23]. Furthermore, through expression profiling studies of immune and metabolic tissues, we found a high level of *Nucb2* in both immune/metabolic tissues and cells, and *Nucb2* expression is responsive to changes in dietary energy intake in adipose derived leukocytes. Based on recent literature and these expression profiling studies of *Nucb2*, we hypothesized that *Nucb2* plays a critical role in crosstalk of immune-metabolic axes. More specifically, such immune-metabolic axis may be involved in the regulation of food intake and consequently insulin resistance and glucose homeostasis. To investigate the role of *Nucb2* in embryonic development as well as in physiological homeostasis, we used a gene targeting homologous recombination technique to design a mouse *Nucb2 loxP*-floxed allele for the generation of a *Nucb2* knockout model.

My dissertation focuses on the roles of innate and adaptive immune system in the pathogenesis of obesity related metabolic disease.

1.2 Metabolism, Obesity and Type 2 Diabetes mellitus

1.2.1 Energy metabolism

Energy metabolism is the general process by which living cells acquire and use the energy needed to stay alive, grow and reproduce. Mammals generate energy from nutrients, including fats, carbohydrates and proteins. Of the three, fat is the most concentrated source of energy, as it provides two times more energy per unit weight than protein or carbohydrates. Metabolic processes are complex, and involve the coupling between the oxidation of nutrients and the

synthesis of high-energy compounds, particularly adenosine 5-triphosphate (ATP), which works as the main energy molecule in all cells.

There are two mechanisms of ATP synthesis; the first involves oxidative phosphorylation through a process by which ATP is synthesized from ADP and inorganic phosphate. The second, substrate-level phosphorylation, is the process by which ATP is synthesized through the transfer of high-energy phosphoryl groups from high-energy compounds to ADP. Both processes occur in the mitochondrion. Indeed, oxidative phosphorylation utilizes the mitochondria's structure, enzymes and energy released by the oxidation of nutrients to form ATP. Substrate level oxidation, however, occurs in the mitochondria via the tricarboxylic acid cycle (TCA) and in the cytoplasm during glycolysis.

Carbohydrate, protein and fat oxidation are the main source of energy in the cell and all converge on the TCA cycle. The beginning of the metabolic pathway for glucose catabolism is glycolysis, which occurs in the cytoplasm. Glycolysis converts glucose to pyruvate and synthesizes ATP. Pyruvate is then transported from the cytoplasm into the mitochondrial matrix. Fatty acids are transported from the cytoplasm to the mitochondrial matrix, with the help of Coenzyme A, to form a temporary compound called acyl-CoA. Acyl-CoA is then converted to acetyl-CoA through β-oxidation reactions, which release electrons that are carried by NADH and $FADH_2$. Amino acids are transported from the cytoplasm to the mitochondrial matrix, and then, broken down in transamination and deamination reactions. The products of these reactions include pyruvate, acetyl-CoA, oxaloacetate, fumarate, alpha-ketoglutarate, and succinyl-CoA, which enter at specific points during the TCA cycle [24].

Different cell types utilize and regulate these catabolic and anabolic energy processes in different manners. Some cell types are more glycolytic, using more glucose, while other cell types utilize more fatty acids in oxidative metabolism. Conceptually, it is easily imagined that certain cells require constant energy sources, whereas others might require fewer or infrequent energy sources, and thus the metabolism of a cell can change over time. For example, there is a clear metabolic difference that exists between classically activated M1 and alternatively activated M2 macrophages. An M1 macrophage is crucial for defense in the initiation of innate immune responses, whereas M2 macrophages play a bigger role within the resolution phase and require much longer-term functions. The difference in metabolic processes between these two related cell types reflects their functions. M1 macrophages undergo aerobic glycolysis upon activation, whereas M2 cells obtain much of their energy from β-oxidation and oxidative metabolism, which can be sustained for longer periods of time [25].

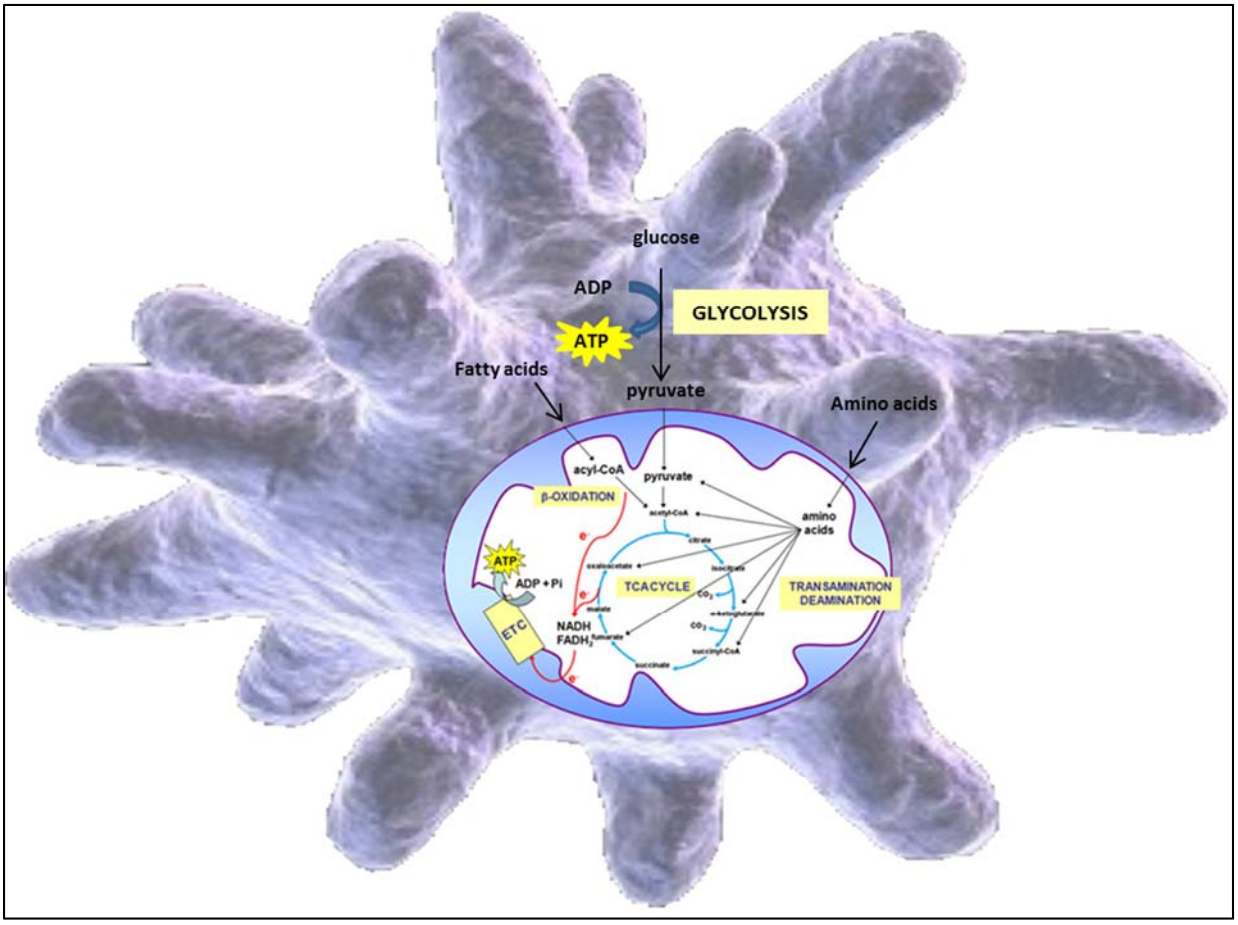


Figure 1.1: Overview of typical energy metabolism processes in a macrophage

A schematic diagram of a macrophage showing how fuel molecules have many different entry points in oxidative metabolism. The smaller dark blue oval contained inside the cell represents the mitochondrion. The mitochondrion has an outer mitochondrial membrane and a folded inner mitochondrial membrane that surrounds the mitochondrial matrix. The ETC (electron transport chain) is represented by a yellow rectangle along the inner mitochondrial membrane. The electrons are transported to the ETC where ATP is synthesized using the protein ATP synthase [24].

For more details on substrate (glucose and lipid) metabolism and cellular respiration, please refer to the appendix.

1.2.2 Obesity

Excess energy intake leads to increased body weight mostly in the form of excess adipose tissue but also with some lipids accumulation in other tissues such as liver, skeletal muscle and heart, all called ectopic fat. The World Health Organization (WHO) defines overweight and obesity as “abnormal or excessive fat accumulation that may impair health”. Obesity is a major risk factor for metabolic disease, diabetes, cardiovascular disease, stroke, arthritis as well as some cancers. The excess fat mass that characterizes overweight and obesity is linked to an

impairment of insulin sensitivity through complex multifactorial mechanisms which are still not fully understood. Adipose tissue dysfunctions have been recognized as essential in this link between weight gain and impairments in insulin signaling [20, 26]. Alterations of fatty acid metabolism leading to increased fatty acid flux cause metabolic disturbances (insulin resistance) in the liver and skeletal muscle [27].

The underlying mechanism behind the cause of obesity, however, is a topic of debate. Obviously, obesity results when energy intake chronically exceeds energy expenditure [28]. However, the contribution of the specific causes to the epidemic of obesity (energy intake vs. energy expenditure) is still very much debated. The role of genetics in body fat regulation is now well established. It is, however, safe to assume that the increased prevalence of obesity has not been due to a genetic change in the Western world in recent history. The recent, large scale rise of obesity must therefore be due mostly to environmental changes, including the availability and composition of the food ingested and changes in physical activity.

1.2.3 Insulin signaling and type II diabetes

Without intervention designed at weight loss, obesity most likely leads to the metabolic syndrome (a constellation of obesity, dyslipidemia, hypertension and insulin resistance) and, in turn, to diabetes mellitus. Diabetes mellitus is defined as a metabolic disorder of multiple etiology, characterized by chronic hyperglycemia, with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both[29]. This disease has become an increasing problem worldwide, and numbers from the WHO show that in 2014, 9% of adults 18 years and older had diabetes. In 2012 diabetes was the direct cause of 1.5 million deaths [30]. The WHO furthermore projects that diabetes will be the 7th leading cause of death in 2030 [31]. The two most common forms of diabetes are type 1 (T1D; autoimmune deficiency in β-cells and insulin secretion) and type 2 (T2D; insulin resistance leading to β-cell failure). The WHO has estimated that 90% of people with diabetes have T2D [30].

Patients with type 1 diabetes require daily administration of insulin, a pancreatic hormone which acts to reduce circulating glucose levels, due to deficient insulin production. T2D, however, is characterized by insulin resistance, and eventual insufficient insulin production. Insulin signaling is required to regulate metabolism. Elevated levels of fasting blood glucose, with subsequent elevations in insulin levels, are often seen in patients with untreated T2D. The body attempts to produce more insulin to overcome the elevated levels of blood glucose as a compensatory mechanism. Failure of insulin signaling in the liver, muscle and adipose tissue leads to hyperglycemia. Over time, T2D results in damage to blood vessels, nerves and leads to internal organ failure [18, 19, 32-35] . Patients with T2D frequently display cardiovascular disease, stroke and other macrovascular diseases. There are also numerous microvascular complications such as microangiopathy in the eyes, kidneys and nerves associated with T2D [36]. Although it was

recorded that diabetes caused approximately 1.5 million deaths in 2012, this number is most likely an underestimation, since many deaths in diabetic patients are documented as heart disease or kidney failure, both common complications of diabetes [30].

A key characteristics of T2D is insulin resistance. This state of resistance induces impaired biological responses to insulin, including decreased glucose transport, glucose oxidation and glycogen synthesis [37]. Insulin resistance has been shown to be partly determined by increased intramyocellular lipid accumulation and reduced muscle fat oxidation [38-41]. Several studies have also observed lower mitochondrial number and activity in type 2 diabetics [42]. In 2008, Turner and Heilbronn [43] published a complete summary of the differences between insulin-resistant and -sensitive individuals. Based on these findings, Samuel et al (2010) postulated that mitochondrial dysfunction would limit the capacity to match fatty acid oxidation to fatty acid (over) supply, leading to increased muscle lipid accumulation and insulin resistance [40].

As presented above, the gradual development of hyperglycemia and diabetes involves numerous pathogenic processes. These include the destruction of the pancreatic β-cells leading to consequent decreased insulin and T2D. Besides ectopic fat causing an inhibition of intracellular insulin signaling, it is now well accepted that insulin receptors can be inhibited by immunomodulatory cytokines such as TNF-α or IL-1β [44]. Classically, insulin resistance from obesity begins in peripheral tissues, such as liver and muscle, thus leading to an increase in blood glucose levels. However, this effect is kept in check by compensation from the pancreatic β-cells that respond to hyperglycemia by increasing insulin secretion [35, 45, 46]. Diabetes is attenuated if increased insulin secretion and action keep pace with the rise in blood glucose. Although the molecular mechanisms that underlie the metabolic impairments of T2D are still largely unknown, insulin signaling can dramatically influence metabolic responses, as suggested by manipulation of components of these signal-transduction pathways in species ranging from flies to humans [47]. It appears, however, that genetic predisposition and environmental factors both play an important role in the onset and risk for development of the disease [48, 49].

Glucose requires glucose transporters (GLUT) for entry into cells, and GLUTs are found throughout all tissues of the body. Moreover, adipose tissue, muscle and liver require insulin for glucose transport into the cells. Adipose tissue insulin-sensitivity, or lack of sensitivity, largely affects whole body glucose homeostasis [50], as adipose tissue is a major regulator of systemic production free-fatty acids but also a consumer of glucose, especially in rodents. It is therefore no surprise that it is considered one of the primary targets for understanding mechanisms mediating the development of insulin resistance and T2D. There are several isoforms of GLUT proteins, and each play a specific role in glucose metabolism [51], however, it is mainly the GLUT4 isoform that is modulated by insulin action in metabolically relevant tissues [52]. A signaling cascade triggered by the activation of the insulin receptor causes translocation of GLUT4 to the

cell membrane [52]. At rest, GLUT4 is sequestered in intracellular vesicles, but fuses with the plasma membrane upon insulin response. The insulin receptor's extracellular α-subunit binds insulin, which leads to an autophosphorylation of the transmembrane β-subunit. Next, the insulin receptor substrate (IRS) is phosphorylated, and can thus interact with Scr-homology-2 (SH2) domains, which in turn activate phosphatidylinositol-3 kinase (PI3K) [47]. Activated PI3K will further activate downstream signaling molecules and proteins like phosphatidylinositol (3,4,5)triphosphate (PIP3), 3 phosphoinositide dependent protein kinase-1 (PDK1) and protein kinase B (Akt/PKB), which ultimately results in translocation of GLUT4 into the membrane [47, 52].

Increased concentrations of insulin cause an increase of GLUT4 proteins in the cell membrane, which consequently increases the glucose transport capacity of cells. Once it has been taken up by GLUT proteins in the cell membrane, glucose is phosphorylated to glucose-6-phosphate through an enzymatic reaction by hexokinase. Glucose-6-phosphate can furthermore be utilized in the glycolytic pathway, *glycolysis*, or be incorporated into glycogen, by glycogen synthase in liver and muscle. Glucose can also be stored as fat via *de novo* lipogenesis in some cases [53].

The changes in glucose metabolism seen in patients with T2D are mostly due to a transition of skeletal muscle, liver and adipose tissue into a state of insulin resistance. Garvey et al. proposed that there is impairment in the translocation of GLUT4 from intracellular stores to the plasma membrane, thus causing insulin's inability to stimulate glucose uptake. This hypothesis has been supported by several studies showing that the cells in tissues and organs of insulin resistant subjects have a surprisingly normal GLUT4 expression, but impaired translocation to the cell membrane [54]. Furthermore, studies on glucose uptake in muscle have also shown reduced activity in IRS-1 phosphorylation and P13K activity in insulin resistant patient with T2D [55, 56]. These data suggest that insulin resistance can occur at many different levels in this pathway and that interference with one or more of these proteins can induce insulin resistance.

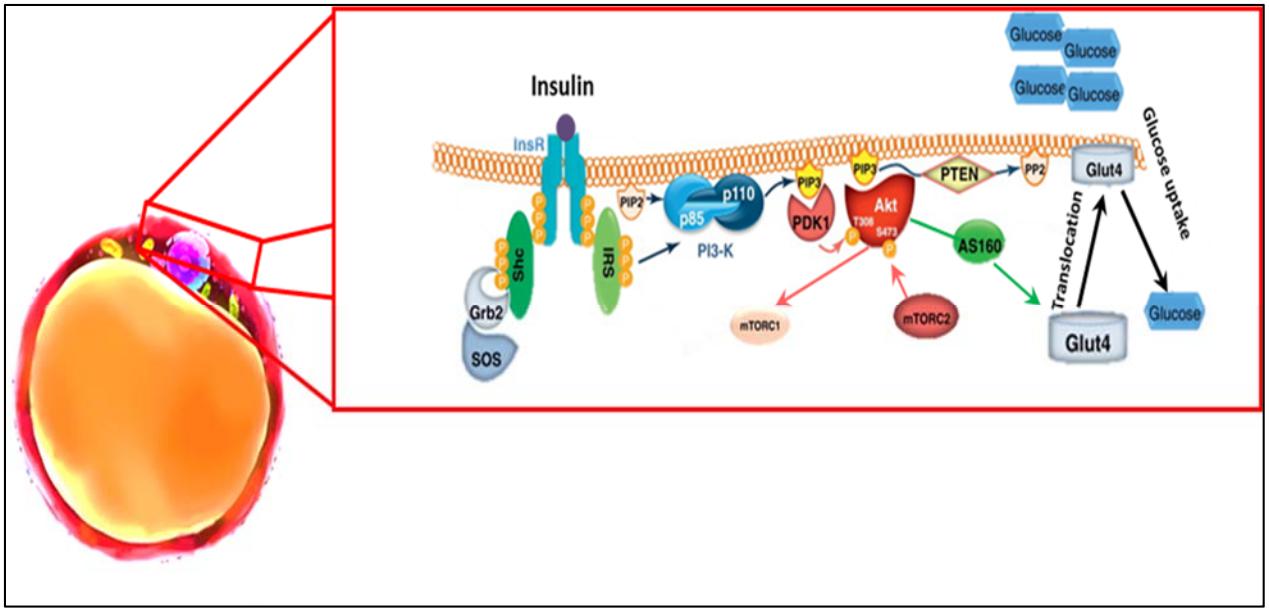


Figure 1.2: Overview of adipocyte insulin dependent glucose uptake via GLUT4 transporter

Insulin binds to the α-subunit of the insulin receptor. This triggers a downstream cascade, which leads to the translocation of GLUT4 to the plasma membrane. The major molecules and proteins are IRS; insulin receptor substrate, PIP_2; phosphatidylinositol (4,5)-biphosphate, PI3K; phosphatidylinositol-3 kinase, PIP_3; phosphatidylinositol (3,4,5)-triphosphate, PDK-1; 3-phosphoinositide dependent protein kinase-1, Akt/PKB; protein kinase B, AS160; Akt substrate regulating GLUT4 translocation.

Below, we are emphasizing the role of a novel emerging mechanism leading to insulin resistance, i.e. local and systemic inflammation.

1.3 Immunity

Every biological organism needs to protect itself against disease to ensure life and evolutionary survival. Immunity is the body's ability to recognize and dispose of substances which it interprets as foreign and harmful to its well-being. While different organisms utilize different mechanisms to safeguard themselves against potential pathogens, immunity broadly requires complex chemical and mechanical activities to defend and protect the body's cells and tissues. These complex processes include the body's ability to protect itself against specific infectious agents and parasites, to accept or reject foreign cells and tissues (such as blood transfusions), to protect against mutations and against cancers (recognition of malignant cells), while all at the same time not attacking normal functioning host cells vital to life. Extensive research has been done into the body's ability to differentiate self-cells, organs and substances from those which are non-self and must be eliminated.

Throughout history, there have been several key observations that led scientists to better understand how we protect ourselves against infections. As early as the plague of Athens in 430 BC, Thucydides' remarkable documentation left not only a clear clinical picture of the pestilence, but most notably described how people who had recovered from a previous bout of the disease conferred at least partial immunity and could nurse the sick without contracting illness a second time [57]. Further observations showed that certain species were immune to some venom, while others succumbed to the infections. From these and other observations, Louis Pasteur constructed a hypothesis that infections by pathogens could be attenuated by exposure to environmental insults, leading to vaccine development and, later, the germ theory of disease [58]. In the 19th century, rapid developments led towards great advances in the mechanism of humoral and cellular immunity.

The immune system protects organisms from infections through a process of layered defenses of increasing specificity. In an overgeneralized manner, physical barriers are the first line of defense preventing pathogens from entering an organism. Our immune systems can be further divided into the innate immune system, which provides an immediate, non-specific response, and the adaptive immune system, which provides a more specific response and is activated by the innate response. These systems have evolved to better protect us from pathogens to avoid infections. Importantly, multiple defense mechanisms have evolved to keep pace with rapidly evolving pathogens. Throughout the day, we are exposed to millions of potential pathogens which the body is able to defend against remarkably well.

1.3.1 Innate immunity

Innate immunity is the first line defense mechanisms that reacts immediately, or within hrs, to many common microorganisms (Figure 1.3). It does not confer long lasting or protective immunity to the host, and is often the only defense in lower animals [59]. The innate immune responses are essential for the control of common bacterial infections. This system mainly consists of macrophages, neutrophils and dendritic cells which provide mechanisms that protect the host from infection by other organisms. Over the past several decades, much has been discovered about the innate immune response to microorganisms. With the identification of pattern recognition receptors (PRR) and pathogen-associated molecular patterns (PAMP), Medzhitov et al. showed that host defense mechanisms are more generic in the response to pathogens via sensors of conserved microbial motifs, rather than each microbe activating a different immune response pathway. Since these findings, many PRRs have been described, and we now know that they are able to recognize several different PAMPs and more recently identified Danger Associated Molecular Patterns (DAMPs). One of the most well studied families of PRRs are the Toll-like family of receptors (TLRs) [60].

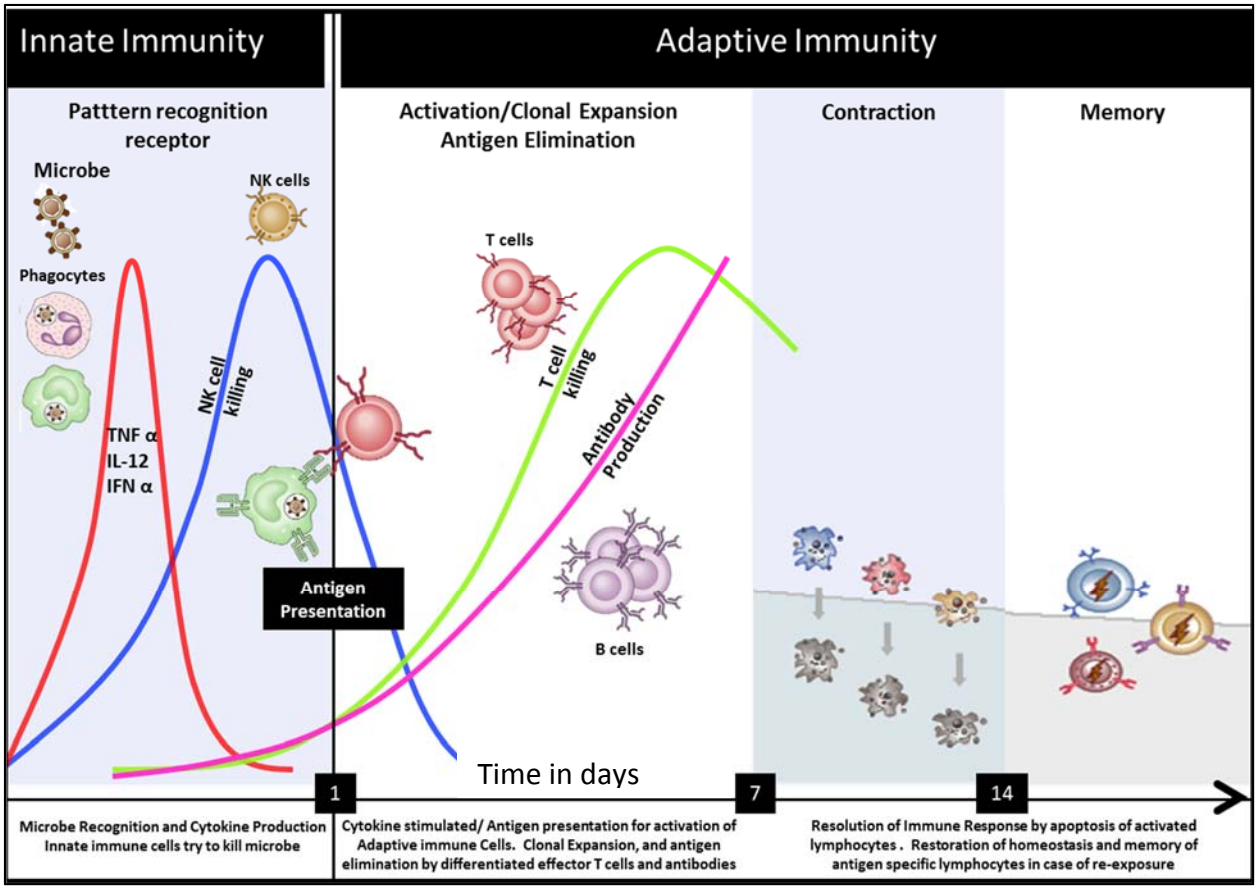


Figure 1.3: Time course of a normal immune response with important cell types

Time-course of a typical immune response beginning with the initial microbe recognition. First, phagocytes of the innate immune system respond and produce chemokines, inflammatory cytokines and antiviral-interferons. These trigger Natural Killer cell responses and complement killing. The phagocytes will then activate the adaptive immune response by antigen presentation to T and B cells. The activated lymphocytes will then undergo clonal expansion with the antigen specific receptor. These receptors can then specifically target microbes in cell mediated T cell killing or humoral mediated antibody production. After resolution of the microbe and immune response, activated lymphocytes will contract by apoptosis. Some cells will retain receptors with antigen specificity and be in a quiescent memory state for possible re-exposure to same microbe.

Innate immunity has numerous functions depending on stimuli. It is essential that the response of the innate immune system be specific to the tissues in the immediate environment of the infection. Firstly, the innate system is important in recruiting immune cells to the sites of infection through the production of chemical factors called cytokines and chemokines. Identification and removal of foreign substances present in organs, tissues, the blood and lymph are next carried out by specialized immune cells called leukocytes. The defense systems of innate immunity are effective in combating many pathogens. However, these defense mechanisms are constrained by the germline-encoded receptors to recognize microorganisms that can evolve more quickly than host cells antigen receptors they infect [61]. The innate immune function is

also important in antigen presentation, in which the innate immune system activates the more long term and specialized adaptive immune system [62].

Inflammation, mediated by the innate immune system, is one of the first immune responses to infection, tissue injury or irritation. Inflammation is stimulated by chemicals released by injured cells and serves to establish a physical barrier against the spread of infection, to promote healing of any damaged tissue, and, finally, to clear the pathogen. The development of acute inflammation is originated by resident immune cells present in all tissues, mainly macrophages and dendritic cells. These cells present PRRs, which recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as PAMPs. At the onset of an infection, burn, or other injuries, these cells undergo activation (one of their PRRs recognizes a PAMP) and release inflammatory mediators, or cytokines, responsible for the cardinal signs of inflammation; swelling (tumor), redness (rubor), heat (calor) and pain (dolor), which were first described in the first century by the Roman scholar Aulus Cornelius Celsus in 1st Century DC [63, 64].

Inflammation is initially the result of the activation of resident macrophages by infectious components, such as lipopolysaccharide acting through toll-like receptors on the macrophages. Most PRRs respond to PAMPs by triggering activation of NFκB, AP1, CREB, C/EBP, and IRF transcription factors [61]. Induction of these genes encoding enzymes, chemokines, cytokines, adhesion molecules, and regulators of the extracellular matrix supports the recruitment and activation of more leukocytes, which are critical for eliminating foreign particles and host debris.

Chemical factors produced during inflammation (histamine, bradykinin, serotonin, leukotrienes, and prostaglandins) sensitize pain receptors, cause vasodilation of the blood vessels and attract phagocytes, especially neutrophils. Cytokines produced by macrophages and other cells of the innate immune system also mediate the inflammatory response. These cytokines include TNF, IL-1β, IL-18 [61]. These cytokines promote leukocyte extravasation by increasing the levels of leukocyte adhesion molecules on endothelial cells [65].

The cells of the innate immune system are critical for the initiation and subsequent direction of the adaptive immune response. They are also important in the removal of pathogens that are targeted by an adaptive response. A critical cell type that links the innate and adaptive immune systems is the dendritic cell (DC). DCs are sentinel cells capable of capturing and presenting antigens to lymphocytes via their major histocompatibility complex (MHC) cell surface proteins. This process mainly occurs in the secondary lymphoid tissues, lymph nodes and the spleen, and is essential for the initiation of the adaptive immune response.

1.3.2 Adaptive immunity

The adaptive immune system, sometimes referred to as the acquired immune system, is a more versatile means of defense, providing increased protection against subsequent reinfection with the same pathogen. The adaptive immune system is composed of B- and T-lymphocytes, and is activated when the infection eludes the innate defense mechanism. These lymphocytes have a greater accuracy than the cells of the innate immune system, and each individual cell responds to a specific antigen present on bacteria, viruses, toxins or foreign objects. The antigen is presented to the lymphocytes by antigen presenting cells via cell receptor MHC. The induction of the adaptive immune system only becomes effective after several days of infection, as this is the time required for antigen-specific T and B cells to locate their specific foreign antigen, proliferate and differentiate into effector cells. After the infection succumbs, the adaptive immune system creates an immunological memory to the specific pathogen, leading to an enhanced response to subsequent infections to the same pathogen, the very pathway utilized by vaccines [62, 66, 67].

Both B and T cells are derived from the same multipotent hematopoietic stem cells. T cell progenitors migrate from bone marrow to the thymus where they develop into T cells, while B cells develop in the bone marrow. It is during development that the cells undergo several selective steps to prevent auto-reactivity to self-antigens. When the developed lymphocytes leave the thymus and bone marrow, they enter the lymphatic system and are considered naïve until they encounter their corresponding antigen. Once a naïve T (or B) cell recognizes the antigen-bound MHC molecule via T (or B) cell receptors and co-receptors, they undergo clonal expansion to rapidly replicate numerous effector cells with the identical antigen recognition receptor. Although they are morphologically similar to one another until they are activated, B and T cells perform different tasks in fighting infections. B cells are vastly important in humoral immune responses, that is, those in the blood, whereas T cells are intimately involved in cell-mediated immune responses [62, 67, 68].

Another central feature of adaptive immunity is the development of immunological memory. This is a process where pathogens are “remembered” through long lived memory B and T cells. When the body is re-exposed to the same antigen, these memory cells proliferate very quickly, producing an immune response that is much faster and much more robust than the initial response to the antigen [62, 66, 67].

1.4 Immuno-metabolism

Historically, immunology and metabolism have been studied separately, but there is increasing evidence of the coupling of immune status to metabolism, and vice versa. A classical observation is that during an immune response, there is often a significant loss of appetite

leading to loss of body weight, suggesting the existence of shared signaling pathways between immune and endocrine systems. More mechanistic studies have shown that many hormones influence immune functions, and similarly many cytokines and growth factors produced during an immune response can have an impact on neuronal and endocrine systems within both a healthy or diseased host [6, 69-75].

While innate and adaptive immunity defend against non-host, invasive pathogens, sterile inflammation is an event that is triggered by a host physical, chemical or metabolic noxiae in the absence of a microbial signal. These non-microbial signals are DAMPs and are sensed by different PRRs. It is now accepted that accumulations of DAMPs, such as necrotic cells, extracellular ATP, saturated fatty acids or free cholesterol crystals, are sensed by PRRs in macrophages which trigger chronic low-grade inflammation [76, 77]. This activates complex inflammasomes, which initiate a sterile inflammatory cascade response, resulting in the release of IL-1β and IL-18 [78]. Such a response is often seen in chronic energy excess and obesity. Sterile inflammation that ensues over time either resolves the initial insult or leads to disease, including metabolic syndrome and insulin resistance [79-83].

Emerging evidence indicates that immune and metabolic interactions control several aspects of metabolic syndrome, aging and chronic diseases. Thymic demise represents a particularly interesting immune-metabolic interaction. Even in metabolically healthy, middle aged individuals, the thymus involutes with deposition of ectopic lipids [84, 85]. In fact, the aging alone is connected with immune-senescence, degeneration of the immune system that is characteristic of a greater susceptibility to infections [86, 87], an inadequate immune response to vaccinations [88, 89] and an increased propensity for autoimmune diseases and cancers [90, 91]. Why and how this occurs remains unresolved. Adipose tissue represents another important site of immune-metabolic crosstalk. It acts as an endocrine organ that not only receives input from other metabolic tissues, but also transmits signals in the form of adipokines, or adipose derived hormones, that act locally and systemically to regulate nutrient balance.

Adipose tissue is now accepted as being heavily influenced by different facets of the immune system, which in turn profoundly affects systemic metabolism [6, 75, 92]. There is ongoing interest in this interaction and much is left to be discovered. Various cell populations of both the innate and adaptive arms of the immune system can resist or exacerbate the development of chronic, low-grade inflammation associated with obesity and metabolic dysfunction [81, 93-95].

Recent studies have shown that metabolic neuro-endocrine peptides and adipokines also control certain aspects of the immune system. This is evident by leptin's ability to affect thymic homeostasis and to act as a proinflammatory cytokine promoting Th1 cell differentiation [96]. There are also reports showing that leptin directly stimulates the expression and release of IL-1α,

IL-1β, IL-6 and TNF-α from both monocytes and T cells [97]. Another example is ghrelin, the endogenous ligand for the growth hormone secretagogue receptor, which has recently been described to be a potent anti-inflammatory mediator both *in vitro* and *in vivo*, and a promising therapeutic agent in treatment of inflammatory diseases and/or injury [98]. Interestingly, these two hormones have important, yet opposite, effects on energy metabolism and inflammation.

1.4.1 Obesity-induced inflammation in adipose tissue and insulin resistance

Obesity-induced inflammation is closely associated with the development of metabolic complications such as insulin resistance and type 2 diabetes [22]. Several immune-signaling receptors and their counterpart ligands are known to be crucial for crosstalk between the innate and adaptive immune system and metabolic pathways. Indeed, the metabolic syndrome is associated with multi-organ inflammatory abnormalities (involving pancreatic, adipose, hepatic, and cardiac and muscle tissue) and represents a major disease burden. TNF-α and IL-1β are postulated to have a negative role in the pathogenesis of many metabolic disorders, as reflected by the testing of a recombinant IL-1 receptor antagonist for the treatment of patients with type 2 diabetes [99].

Adipose tissue in rodents and humans is composed of different "types" of fat. They can be compartmentalized into two main categories: visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). Accumulation of VAT is associated with insulin resistance and a high risk for the development of the metabolic syndrome and type 2 diabetes. Conversely, accumulation of SAT has been shown to have a possible protective effect against these metabolic abnormalities, especially when distributed in the gluteo-femoral region of the body (lower body obesity). In obese individuals, VAT has a higher percentage of large adipocytes and greater numbers of inflammatory cells when compared to SAT [100, 101]. High fat feeding increases the numbers of adipocyte precursor cells and mature adipocytes in both VAT and SAT. Similar to increases in adipocytes, non-adipocyte precursor cells, such as stromal vascular cells, also increase in high fat feeding conditions. Although this stromal vascular fraction shows dramatic increases in VAT after 2-4 weeks of a high fat diet (HFD), this is not seen as dramatically in SAT [102]. It is also now accepted that mice fed HFD recruit both innate and adaptive immune cells in their adipose tissue as they gain weight [103, 104]. Adipose tissue leukocytosis and chronic inflammation is now accepted to play a profoundly deleterious effect on the pathogenesis of obesity induced metabolic syndrome and type 2 diabetes.

Stephens and Pakala (1991) showed that the proinflammatory cytokine TNF-α was sufficient to elicit a phenotype of insulin resistance by repressing GLUT4 and transcription factors C/EBP family in 3T3-L1 adipocytes [75]. Hotamisligil et al further made early connections between obesity, inflammation and insulin resistance, reporting an induction of TNF-α in adipose tissue from four rodent models of obesity and diabetes. They went on to demonstrate that neutralizing

TNF-α in obese rats ameliorated insulin resistance [6, 92]. Since this finding, follow up studies have shown that alterations in the immune system during chronic over nutrition can result in low-grade inflammation, which favors the development of insulin resistance [21, 105]. Similar to studies in rodents, obese individuals with excess adipose tissue show increased circulating levels of the proinflammatory marker C-reactive protein. Moreover, increased levels of C-reactive protein, IL-6, and IL-1β are predictive of the development of type 2 diabetes in various populations [106].

In summary, it is now evident that obesity triggers the accumulation of immune cells, mostly macrophages, in the adipose tissue resulting in local sterile inflammation and ultimately systemic insulin resistance.

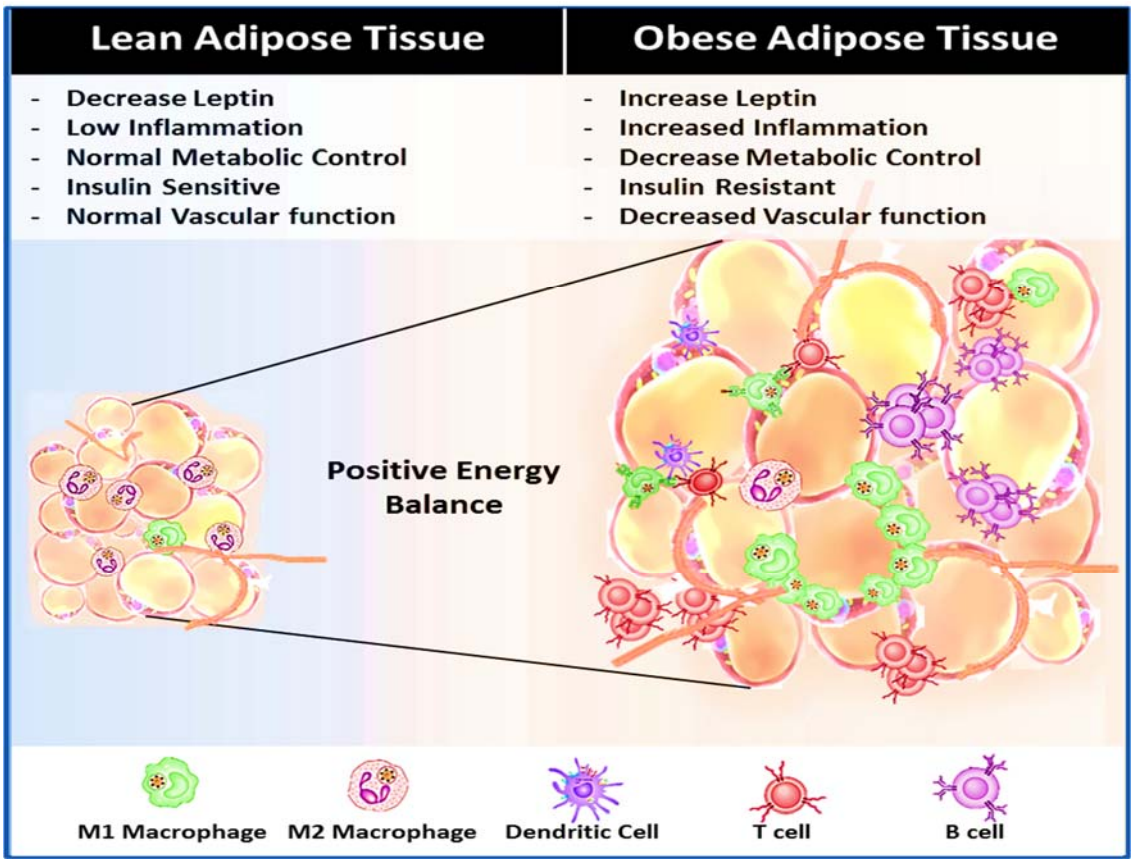


Figure 1.4: Adipose tissue expand during obesity in parallel to inflammation and metabolic dysregulation

Excess energy leads to adipose tissue expansion by both hypertrophy (larger adipocytes) and hyperplasia (more adipocytes). With these hypertrophic adipocytes comes secretion of chemoattractant, leading to adipocyte leukocytosis. Increases in activated immune cells results in increases in proinflammatory cytokine secretion. This results in polarization of macrophages to a proinflammatory M1 phenotype and drives inflammatory lymphocyte infiltration. Augmented lipolysis leads to increased levels of free fatty acids. This micro-environment negatively impacts the insulin signaling pathway and leads to an insulin resistant state and dysregulated glucose homeostasis.

1.4.2 Type I interferons, inflammation and glucose metabolism

Interferons (IFN) are a group of polypeptides that have antiviral, anti-proliferative, cytostatic, antitumor and immunomodulatory effects on the innate and adaptive immune response [107, 108]. IFNs cause changes in cell membranes, enzyme metabolism and protein synthesis. Awareness of the existence of more than one type of IFN advanced progressively as a result of the molecular cloning of the different IFN genes. Since the first published description of IFN [109, 110], there has been an explosive growth in our understanding of genes encoding the IFNs and their receptors, their complex signaling cascades and regulation, and their biological activities [111]. IFNs are typically classified as Type I (IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω) and Type II interferons (IFN-γ), and more recently Type III interferons [112]. There has recently been an increase in scientific and clinical interest in elucidating the biology of type I interferons, which began approximately 60 years ago with the discovery of interferons and concept introduced by Jean Lindenmann of "viral interference," a property that reduces the ability of a virus to infect cells [109, 110].

Type I interferon is a sophisticated early defense mechanism positioned to combat microbial infections and assist in the initiation of the adaptive immune response. By evolutionary selection, the multiple type I IFN species evolved to signal through the same IFN-α/β receptor. The interferon alpha gene cluster consists of 14 IFN-α homologous genes in mice and 13 in humans, as well as one IFN-β gene. The evolutionary similarities of type I IFN among many animal species attests to the necessity of type I interferon response for survival. The function of these various species and the need for their differing physical profiles, receptor affinities and downstream gene activation/silencing capabilities is, however, largely unknown. Type I interferon is now a common therapy for autoimmune and inflammatory disorders, yet the mechanism of its action remains poorly understood.

In the last decade, particular attention has been paid to the anti-inflammatory effects exerted by type I IFN [113, 114]. A number of studies have shown the effectiveness of this family of cytokines in reducing inflammation in different experimental settings. Most importantly, type I IFN is successfully used in the clinic not only for the treatment of diseases of viral origin, but also for managing diseases such as arthritis and multiple sclerosis [115-119]. Recently, evidence has shown that type I interferon is an inhibitor of IL-1β production and inflammasome activation [120]. Type I interferon signaling, via the STAT1 transcription factor, represses the activity of the NLRP3 inflammasomes, and thereby suppresses caspase-1 dependent IL-1β. It has further been shown that type I IFN treatment in monocytes obtained from patients with multiple sclerosis leads to the production of substantially less IL-1β than monocytes derived from healthy donors.

In addition to the ability of type I interferons to inhibit the production of proinflammatory cytokines, several papers suggest that type I interferons influence metabolic responses both

centrally and peripherally. Studies show that IFN-α could have regulatory effects on body temperature and food intake [121, 122]. Furthermore, there is evidence that IFN-β induces glucose uptake in cells [123]. More specifically, evidence suggests a biphasic IFN-β-inducible uptake of glucose by cells, mediated by phosphatidylinositol 3-kinase (PI3K)/Akt, and IFN-β-inducible regulation of GLUT4 translocation to the cell surface. This data reveals that type I interferons regulate glucose metabolism mediated by signaling effectors in a manner similar to insulin. Furthermore, a commonly prescribed diabetic drug, metformin, enhances antiviral effects of type I interferon during infection, suggesting a clear necessity for glucose availability/utilization and proper immune response.

1.5 Nucleobindin-2

Nucleobindin (Nuc) was first identified in B-lymphocyte cell lines and is proposed to have DNA binding domains, suggesting it may function as a transcription factor [124]. Nucleobindin is a Golgi-resident protein that plays a key role in calcium homeostasis in the Golgi network [125]. Nucleobindin-2, or NUCB2/NEFA (for DNA binding/EF-hand/acidic protein), is a calcium binding protein which also contains nuclear targeting signals, 2 helix-loop-helix regions with 2 concurrent EF-hand motifs and a leucine zipper motif [126] (Figure 1.6). More recently, *Nucb2* has been shown to be present in the endoplasmic reticulum and in mitochondria [127]. NUCB2 is encoded on chromosome 7F1, containing 14 exons in mice (chromosome 14 in human) with a genomic sequence of 36,186 bps. Post transcription modifications leads to an mRNA sequence of 1,697 bp with a 420 amino acid long polypeptide. This polypeptide then undergoes posttranslational modifications and is cleaved into three peptides termed nesfatin-1, nesfatin-2 and nesfatin-3 (Figure 1.5).

Nesfatin-1, the 82-amino acid peptide derived from *Nucb2*, was first described in 2006 to be a regulator of food intake and energy expenditure [14] via regulation of the melanocortin signaling pathway in hypothalamic nuclei. In 2008, it was further shown to have a much wider distribution in the brain [128]. Daily intra-cerebroventricular (i.c.v.) injection of nesfatin-1 decreased food intake in a dose-dependent manner, whereas injection of an antibody neutralizing nesfatin-1 stimulated appetite [14]. Moreover, under starved conditions, its expression was decreased in the hypothalamic paraventricular nucleus. Chronic i.c.v. injection of nesfatin-1 reduce body weight, whereas rats gain body weight after chronic i.c.v. injections of an antisense morpholino oligonucleotide against the gene encoding *NUCB2,* leading the authors to conclude that nesfatin-1 is a potent satiety signal and anorexigenic peptide hormone with potential clinical applications in obesity treatments [14, 129-131].

Intriguingly, more recent experimental evidence suggests that *NUCB2*/nesfatin-1 is ubiquitously expressed in peripheral tissues including the pancreas, adipose tissue, liver, kidney

and the gut, in addition to the brain [132-134]. Such distribution implies that *NUCB2*/nesfatin-1 has important metabolic as well as immunologic functions. Recent reports indicate that *Nucb2*, or nesfatin-1, also plays important roles in physiological processes beyond the control of feeding. For example, two studies suggest that nesfatin-1 is involved in glucose homeostasis, gastrointestinal function, water intake, temperature regulation and sleep [135, 136]. These pleiotropic actions emphasize the physiological relevance of *Nucb2* and/or nesfatin-1 to metabolism. However the mechanisms underlying the involvement of these proteins in whole body metabolism are still unknown.

Darambazar et al (2015) recently showed that treatment with leptin *in vivo* and *in vitro* markedly increased *Nucb2* mRNA expression in the paraventricular nucleus of the hypothalamus (PVN), a brain region with critical roles in the control of ingestive behavior. The authors further went on to show that peripheral and central injections of leptin failed to significantly inhibit food intake in mice receiving adeno associate virus (AAV)-*NUCB2*. These results indicate that PVN *NUCB2*/nesfatin-1 is directly targeted by leptin, and mediates its anorexigenic effect [137]. More recent studies reported positive correlations in hypothalamic *Nucb2* mRNA expression and serum leptin levels in rats [138]. Moreover, *Nucb2* expression in white adipose tissue is thought to be regulated by the hypothalamus via the sympathetic nervous system [133]. In addition to these findings, others showed that nesfatin-1 crosses the blood-brain barrier [139, 140] and that peripheral administration of nesfatin-1 can decrease food intake in mice [23].

These initial findings provoked much excitement among groups studying obesity and energy metabolism. An important and unresolved question, however, is whether altered nesfatin-1 or *Nucb2* activity in the hypothalamus contributes to insulin resistance in obesity. Conditional *in vivo* targeting of *Nucb2* to different brain regions or peripheral tissues would also be informative of function, however technical challenges arise as nesfatin-1, nesfatin-2 and nesfatin-3 might play differing functions yet are encoded by the same gene. Finally, the nesfatin-1 receptor remains unknown, making studies determining precise sites of peptide action difficult.

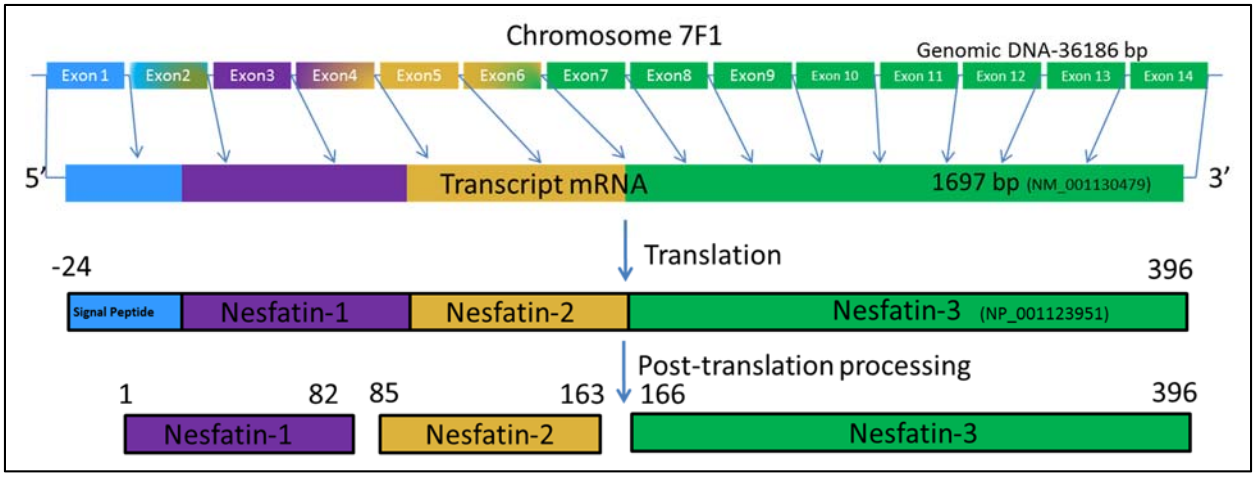


Figure 1.5: Graphical representation of Nucleobindin-2 gene, transcript and proteins

Nucb2 is transcribed from a 14 exon, 36,186 base pair sequence to a 1,697 mRNA sequence. It is initially translated into a 420 amino acid molecule, comprising a signal peptide and Nesfatin-1,-2 and -3 sequence. The signal peptide (24aa's) is removed from the amino terminus. Nesfatin-1, -2 and -3 can then be post-translationally proteolytically cleaved to form the 3 derivative peptide products.

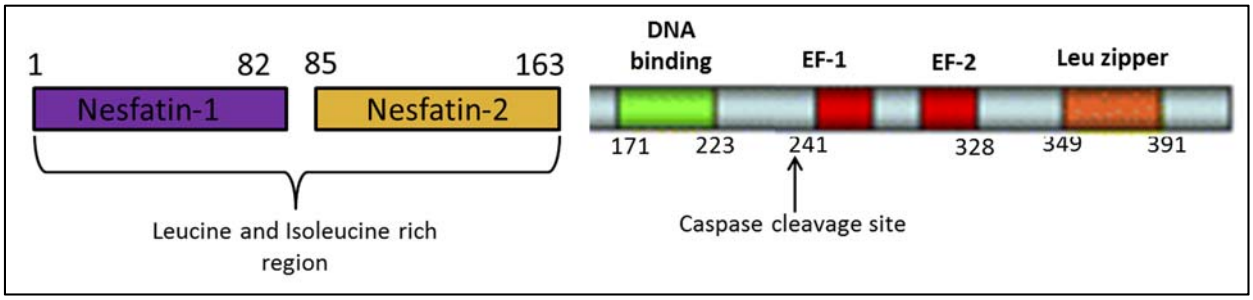


Figure 1.6: Post translational peptide proteins and peptide motifs

Nucleobindin-2 peptide cleaved domain structure. Nesfatin-1 and Nesfatin-2 have leucine and isoleucine rich regions. Nesfatin-3 contains a DNA binding domain (DNA), two helix – loop – helix EF-hand domains (EF-1 and EF-2), a leucine zipper motif (LZ) and a C-terminal domain (C-term).

After this overall introduction to the importance of an immune-metabolic axis in metabolic health, I will now describe in the next 3 chapters the experiments that I designed and performed to assess the impact of nucleobindin-2 (*Nucb2*) and Nesfatin-1 on metabolic pathways and metabolic health. The final chapter will be a discussion of the experiments conducted and how they fit within scientific literature on immune-metabolic interactions with some suggested future studies to better understand the field of immune metabolism.

Chapter 2: Effects of Nucleobindin-2 on Glucose Homeostasis and Insulin Resistance

2.1 Introduction

As previously mentioned in Chapter 1 above, prior studies have found that *Nucb2* pharmacologically decreases food intake by regulating melanocortin signaling in the hypothalamus [14]. Moreover, nesfatin-1 has recently been reported to be dramatically upregulated and secreted into the culture media during the differentiation of 3T3-L1 preadipocytes into adipocytes [134]. This group further showed that treatments with TNF-alpha, IL-6, insulin, and dexamethasone also increased nesfatin-1 secretion. In addition, circulating nesfatin-1 levels were higher in high-fat-fed obese mice and showed positive correlation with body mass index in humans. These studies induced our interest in the *Nucb2* gene.

Additionally, several studies have recently suggested positive correlations in plasma nesfatin-1 levels in patients with T2D and is associated with BMI, plasma insulin, and insulin resistance [141-144]. Yang *et al*. recently indicated that infusion of nesfatin-1 into the third cerebral ventricle markedly inhibited hepatic glucose production, promoted glucose uptake and was accompanied by decreases in hepatic mRNA and protein expression of PEPCK in both chow and HFD fed rats [144]. Fascinatingly, they went on to show that nesfatin-1 increased insulin receptor, IRS-1, AMPK, and Akt and resulted in an increased Fos in the hypothalamus that mediated glucose homeostasis.

Furthermore, we have observed that *Nucb2* is highly expressed in cells of both innate and adaptive immune system, but the function of this gene in immune-response or obesity-associated metabolic dysfunction is still largely unknown (Figure 2.6). In light of the fact that injections of nesfatin-1 create an anorexic phenotype, we hypothesized that a complete genetic knockout of *Nucb2* would have an opposing effect and would lead to hyperphagia and obesity. We also were interested in determining if *Nucb2* directly interfered with insulin signaling. We also sought to determine the causal and physiological role of Nucb2 in the regulation of immune-metabolic crosstalk and inflammation *in vivo*. In order to do so we generated a Nucb2 floxed allele by introducing a PL253-loxP-frt-neo cassette to exon 3. We initially created a global knockout by crossing floxed Nucb2 founder C57Bl/6 mice with a global Ella-^{Cre} driver to remove the floxed sequence (Figure 2.3, Figure 2.4, and Figure 2.5). Overall, the most striking phenotype in the mice lacking *Nucb2* is significant impairments in insulin sensitivity and glucose homeostasis in high fat fed-induced obesity.

2.2 Materials and Methods

2.2.1 Mice and animal care

Mice were generated with the transgenic core at Pennington Biomedical Research Center by Dr. Randall Mynatt and kept in a pathogen-free barrier facility maintained at 22–24°C with a 12:12-h dark-light cycle (lights on at 0700 h). Initial genotyping was done with help by Dr. Bolormaa Vandanmagsar. Mice were housed up to 5 per cage and given *ad-libitum* access to normal chow (#5002, at least 4.5% kcal crude fat, LabDiet), low fat diet (LFD; #D12450B, 10% kcal as fat, Research Diets, New Brunswick, NJ) or a high fat diet (HFD; #D12492i, 60% kcal as fat; Research Diets, New Brunswick, NJ) and sterilized water (Hydropac Alternative Watering System: Seaford, DE, USA). The mice were placed on the LFD or HFD at 6 weeks of age and maintained on it until death. All transgenic and WT mice were cross-fostered to parent cohorts in our colony. The sentinel mice in our animal rooms were negative for currently tested standard murine pathogens (ectromelia, epizootic diarrhea of infant mice [EDIM], lymphocytic choriomeningitis [LCMV], Mycoplasma pulmonis, mouse hepatitis virus [MHV], murine norovirus [MNV], mouse parvovirus [MPV], murine minute virus [MVM], pneumonia virus of mice [PVM], reovirus type 3 [REO3], Theiler's murine encephalomyelitis virus [TMEV], and Sendai virus) at various times while the studies were performed. Changes in body weights were measured during the life of the animals. Fat mass (FM) and fat free mass were assessed by time-domain-nuclear magnetic resonance (Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany) at different time points.

2.2.2 Hyperinsulinemic-euglycemic clamp

Clamp studies were performed according to recommendations of the Mouse Metabolic Phenotyping Center Consortium [145]. The clamp studies were performed on 14 week old, chow-fed mice, and on 12 week old mice (n=8 wild type, n=7 *Nucb2* -/-) on a high fat diet for 6 weeks (n=11 wild type, n=7 *Nucb2* -/-). After surgical implantation of an indwelling catheter in the right jugular vein, mice were allowed to recover for 7 days prior to the clamp experiments. Following an overnight 14-h fast, mice were infused with 3-[^{3}H] glucose (HPLC purified; PerkinElmer Life Sciences, Waltham, Massachusetts) at a rate of 0.05 µCi/min for 120 min to determine basal glucose turnover. Next, a primed infusion of insulin and 3-[^{3}H] glucose was administered for 4 min at rates of 7.14 milliunits·kg−1·min−1 and 0.24 µCi/min, respectively, after which the rates were reduced to 2.5 milliunits·kg−1·min−1 insulin for chow fed mice/3 milliunits·kg−1·min−1 insulin for HFD mice (Novolin; Novo Nordisk, Bagsværd, Denmark) and 0.1 µCi/min 3-[^{3}H] glucose for the remainder of the experiment. Plasma samples were obtained from the tip of the tail at 0, 25, 45, 65, 80, 90, 100, 110, 120, 130, and 140 min for plasma glucose, insulin, and tracer levels. A variable infusion of 20% dextrose was given to maintain euglycemia (100-120 mg/dL). Also, mice received an i.v. albumin-containing solution mimicking artificial plasma (115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl^2$-$6H^2O$, 1.2 mM NaH^2PO4-H^2O, 1.2 mM Na^2SO^4, 2.5 mM CaCl-$2H^2O$, 25 mM

$NaHCO^3$, and 4% BSA [pH 7.4]) at a rate of 4.2 µL/min, during the insulin-stimulated period of the clamp to compensate for volume loss secondary to blood sampling.

Glucose turnover was calculated as the ratio of the 3-[^{3}H] glucose infusion rate to the specific activity of plasma glucose at the end of the basal infusion and during the last 40 min of the hyperinsulinemic-euglycemic clamp study. Hepatic glucose production represents the difference between the glucose infusion rate and the rate of glucose appearance. A 10-µCi bolus injection of 2-deoxy-d-[^{14}C]-glucose was given at 90 min to determine tissue-specific glucose uptake, which was calculated from the area under the curve of 2-deoxy-d-[^{14}C]-glucose detected in plasma and the tissue content of [^{14}C]2-deoxyglucose-6-phosphate, as previously described [146]. Following collection of the final blood sample, mice were anesthetized with an intravenous injection of 150 mg/kg pentobarbital, and tissues were harvested and frozen with aluminum forceps in liquid nitrogen. All tissues were stored at −80 °C until later use.

2.2.3 Serum hormones and metabolite profiles

Blood was collected from all mice by cardiac puncture following CO^2 administration. Blood collected from mice was allowed to clot at 22°C for 2-h before centrifugation at 2,000g for 20 min at 4°C. Serum was kept at -80°C until assays were performed. All assays were performed in accordance with the provided standard operated procedures. Insulin levels were measured using the Mercodia Ultrasensitive Mouse Insulin ELISA (Mercodia; Uppsala, Sweden). Adiponectin was measured using Mouse Adiponectin ELISA kit (EMD Millipore; St. Charles, Missouri, USA). Leptin was measured using Mouse/Rat Leptin Quantikine ELISA (R&D Systems; Minneapolis, MN, USA). IL-18 was measured using MBL mouse IL-18 ELISA (Medical & Biological Laboratories; Nagano, Japan). Nesfatin-1 was measured using nesfatin-1 (1-82aa) (rat) EIA kit (EK-003-22; Phoenix Pharmaceuticals; Burlingame, CA, USA) with a detectable range of 0.1-1000ng/ml. *Nucb2* was measured using mouse Nucleobindin-2 (*NUCB2*) ELISA kit (CUSABIO; Wuhan, Hubei Province, P.R. China) with a detectable range of 15.6-1000 pg/ml. For the nesfatin-1 and *Nucb2* assays, some sample diluent was spiked with nesfatin-1, nesfatin-2 and nesfatin-3 recombinant protein at concentrations of 100 nM (Phoenix Pharmaceuticals; Burlingame, CA, USA). Blood glucose was detected using Breeze2 Blood glucose test strips (Bayer HealthCare; Mishawaka, IN, USA) via tail bleed.

2.2.4 Tissue digestion and positive selection of T cells and macrophages

Adipose tissue was digested using enzymatic digestion as previously described [147]. Adipose tissue stromal vascular fraction cells were then collected, and T cells and macrophages were positively selected using CD3 and F4/80 labeled antibodies (Dynabeads, Life Technologies).

2.2.5 Quantitative PCR

RNA from tissue and cells was isolated using an RNeasy Plus mini and micro kit (Qiagen; 74106 and 74034) according to the manufacturer's instructions. DNA digestion was performed

on the columns to remove DNA using RNase-Free DNase according to manufacturer's instructions (79254; Qiagen). Following RNA purification, samples were then used for iScript cDNA synthesis using a reverse transcriptase PCR kit (BIO-RAD; Hercules, CA, USA). Quantitative PCR was performed with the LightCycler 480 II (Roche Applied Science; Indianapolis, IN, USA) and Power SYBR Green detection reagent (Applied Biosystems by Thermo Fischer Scientific; Woolston Warrington, UK). Primer sequences for transcripts encoding proteins involved in lipid and glucose metabolism were designed with Primer Express Software. For *Nucb2*, we used the forward primer 5'-AAAACCTTGGCCTGTCTGAA-3' and the reverse primer 5'-CATCGATAGGAACAGCTTCCA-3'. In all qRT-PCR experiments, 25 ng cDNA was used. Fold induction of gene expression with *Nucb2* was analyzed with the ΔΔCt method (also known as the comparative Ct method) as determined by the following equation: ΔΔCt = ΔCt treatment (age and diet) - ΔCt control (young/chow). Here, the ΔCt is the Ct value for the sample (*Nucb2*) normalized to the endogenous housekeeping gene *GAPDH* transcript.

2.2.6 Tissue Hematoxylin and Eosin (H&E) staining

The adipose tissue and liver were collected from mice, fixed in 4% (vol/vol) buffered paraformaldehyde, embedded in paraffin and optimal cutting temperature (OCT) compound, then cut into 5 µm thick sections. Tissue sections were stained with hematoxylin and eosin (H&E). Fluorescent images of H&E-stained paraffin-sections were photographed using the Texas Red filter cube on an Axioplan 2 imaging microscope (Carl Zeiss Microscopy; Thornwood, NY, USA). Quantification of adipocyte size was measured using ImageJ image analysis software. The averages were found by measuring 15 adipocytes from 3 different sections from 4 different mice per group.

2.2.7 Statistical analysis

We used a two-tailed Student's *t*-test to examine differences between genotypes or treatments (age and feeding) with a $P < 0.05$ considered statistically significant. The results are expressed as the arithmetic means ± SEM. Differences between means and different genetic model were determined by one-way ANOVA using Tukey's test, using SigmaStat or Graphpad Prism software, which protects the significance ($P < 0.05$) of all pair combinations.

2.3 Results

2.3.1 *Nucb2* gene expression in immune cell subsets is regulated by diet

We first sought to understand if *Nucb2* expression was related to either age and/or diet composition. We performed qRT-PCR using 12.5 ng cDNA to measure mRNA levels of *Nucb2* from 3 month and 6 month old wild type mice either on low fat diet or a high fat diet (n=6 per group). There were no significant differences in *Nucb2* expression levels due to age or diet in the

hypothalamus, visceral adipose tissue, and in liver (Figure 2.1). Inguinal adipose tissue on the other hand, showed differences in *Nucb2* expression. At age 3 months, HFD increased *Nucb2* expression levels (LFD and HFD: 1 ± 0.03 and 3.04 ± 0.51, respectively, p=0.003); however HFD had no differences in *Nucb2* expression levels at 6 months of age (LFD and HFD: 1.39 ± 0.38 and 0.92 ± 0.24 respectively, p=0.31). A decrease in *Nucb2* expression levels was observed with aging (3 month and 6 month: 3.04 ± 0.43 and 0.92 ± 0.24, respectively, p=0.001) only on a HFD (Figure 2.1).

In the pancreas, we saw significant changes in *Nucb2* gene expression in response to aging. HFD mice had a trend towards a decrease in *Nucb2* expression levels at both 3 and 6 months of age; however, this was not significant (3 month LFD and HFD: 1 ± 0.12 and 0.63 ± 0.12, p=0.06 respectively and 6 months LFD and HFD: 0.42 ± 0.06 and 0.28 ± 0.05 respectively, p=0.12) (Figure 2.1). Aging, on the other hand, induced a significant decrease in pancreatic *Nucb2* expression levels in both LFD (3 month and 6 month: 1.0 ± 0.12 and 0.42 ± 0.063, respectively, p=0.003) and HFD (3 month and 6 month: 0.629 ± 0.12 and 0.28 ± 0.05, respectively, p=0.02). This suggests *Nucb2* expression is downregulated in aging and obesity.

Interestingly, diet composition regulated the expression of *Nucb2* in both macrophages and T lymphocytes isolated from adipose tissue. Positively selected macrophages from visceral adipose tissue showed a significant increase in *Nucb2* expression in HFD mice aged 3 months (LFD and HFD: 1.0 ± 0.19 and 6.08 ± 1.35, respectively, p=0.04) and 6 months (LFD and HFD: 2.47 ± 0.24 and 7.31 ± 0.9, respectively, p=0.007) (Figure 2.2). Additionally, purified T cells from visceral adipose tissue displayed an increase in *Nucb2* expression levels in HFD mice aged 3 months (LFD and HFD: 1.0 ± 0.06 and 1.81 ± 0.13, respectively, p=0.003) and 6 months (LFD and HFD: 0.70 ± 0.10 and 1.38 ± 0.11, respectively, p=0.006) (Figure 2.2).

Overall, HFD stimulates increased expression of *Nucb2* in isolated macrophages and T cells from visceral adipose tissue. HFD also significantly increases *Nucb2* expression in 3 month old mice; however, this is not seen at 6 months of age. *Nucb2* in the pancreas, on the other hand, is significantly reduced by age in both chow conditions and HFD conditions. It is unclear, however, if these changes are a result of the diet composition directly, or obesity and weight gain.

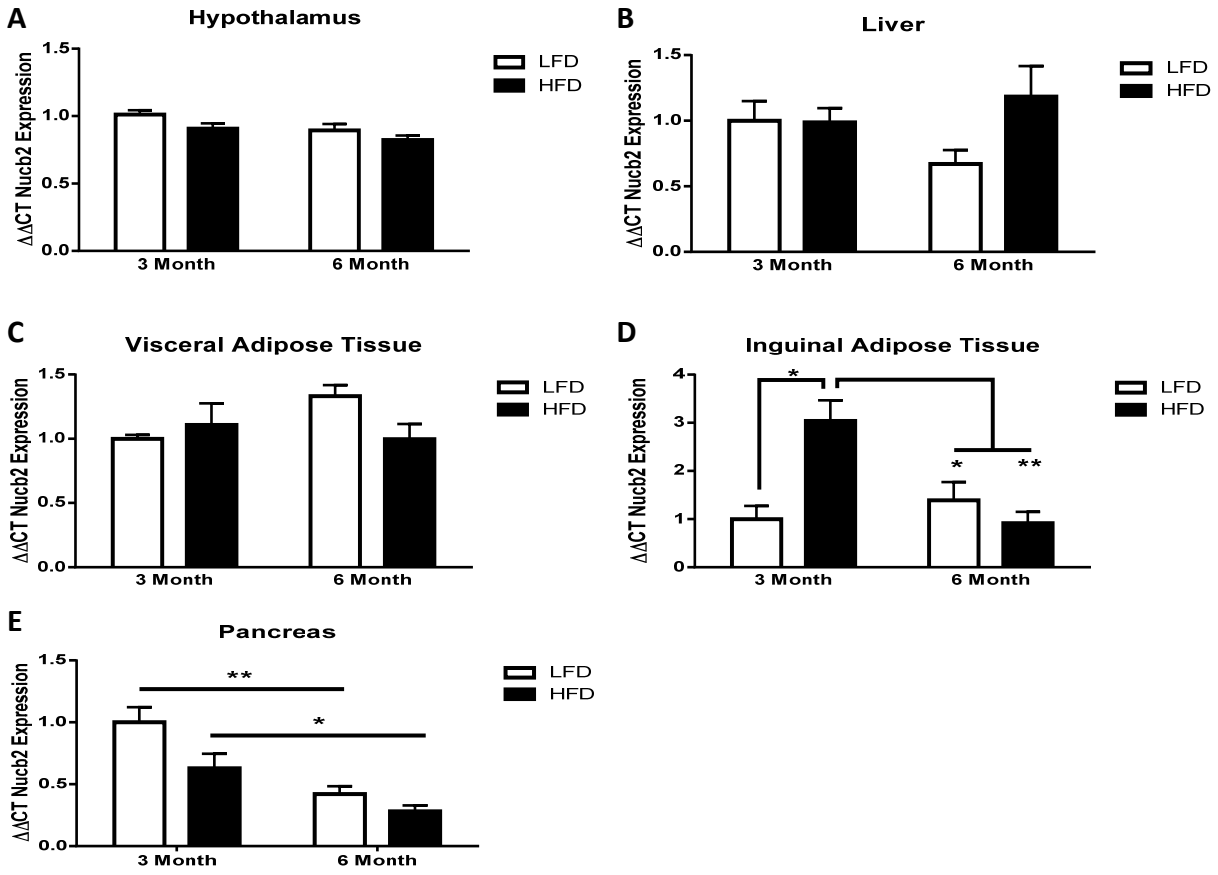


Figure 2.1: Age and diet modulates *Nucb2* expression differently in different tissues

Relative *Nucb2* gene expression (mRNA) levels were measured by quantitative RT-PCR. Values are expressed as fold change means ± S.E.M., with mean expression in 3 month old LFD as control (n=6 per group). (A) Hypothalamus (B) Liver (C) Visceral Adipose Tissue (D) Inguinal Adipose Tissue (E) Pancreas. *p< 0.05, **p<0.005.

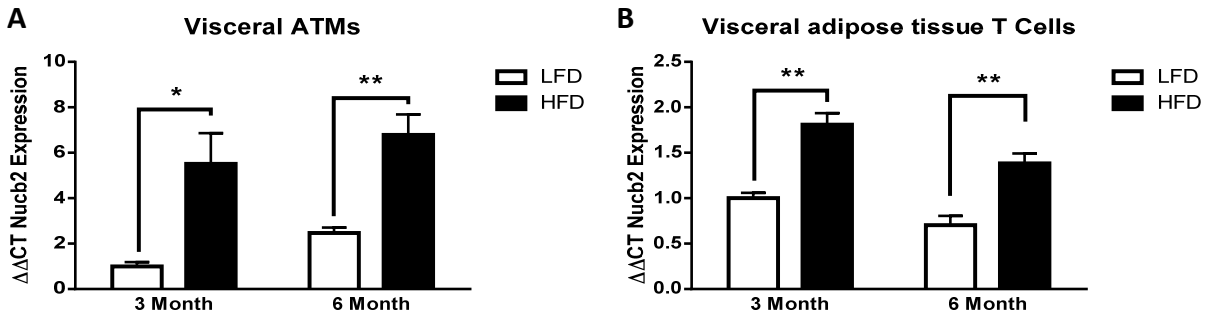


Figure 2.2: High fat diet-induced obesity increases *Nucb2* expression in visceral adipose tissue macrophages (ATMs) and T cells

Relative *Nucb2* gene expression (mRNA) levels were measured by quantitative RT-PCR from positively selected macrophages (F4/80+) and T cells (CD3+). Values are expressed as fold change means ± S.E.M., with mean expression in 3 month old LFD as control (n=6 per group). (A) Visceral Adipose Tissue Macrophages (B) Visceral Adipose Tissue T Cells. *p< 0.05, **p<0.005.

Nucb2 target sequence, vector construct, genotyping, and creation of a global *Nucb2 -/-* mouse

Combined with prior studies suggesting strong effects of nesfatin-1 on energy intake and body composition, our findings that *Nucb2* is highly expressed in both innate and adaptive immune cell subtypes and is regulated by diet lead us to hypothesize that *Nucb2* may play important roles in immune-metabolic interactions. The function of this gene in immune-response or obesity-associated metabolic dysfunction, however, is still largely unknown (Figure 2.6). We thus sought to create a *Nucb2* knockout mouse to examine its function in the context of immune-metabolic function. Considering injections of nesfatin-1 create an anorexic phenotype [14], we reasoned a complete knockout would have an opposing effect and lead mice to be hyperphagic and become obese.

We used a genetic targeting approach to investigate the physiological role of *Nucb2* in the regulation of immune-metabolic crosstalk, and in inflammation *in vivo*. We did this by generating a *Nucb2* floxed allele by recombination of a PL253-loxP-frt-neo cassette to exon 3 in mouse embryonic stem cells (

Figure 2.3). These mice were created by the Pennington Biomedical Research Center transgenic core with Dr. Randy Mynatt using established technology described in herein. Once our vector was correctly designed and screened against neomycin for initial enrichment of the targeted clone, we used these embryonic stem cells for injection into blastocysts for the generation of heterozygous Nucb2-loxP floxed mice. These positive ES cells were used. Using polymerase chain reaction to genotype these neo-founder mice (Figure 2.4), we then removed the neomycin resistant drug marker by crossing to a recombinase FLP derived mouse which recognizes the flippase recognition target (FRT) for mediated cleavage and generation of our founder mice (Figure 2.5). We initially created a global knockout by crossing floxed Nucb2 founder C57Bl/6 mice with a global Ella-^{Cre} driver to remove the floxed sequence.

We initially confirmed expression of Nucb2 in immune cell subsets (B cells, T cells and macrophages) as well as different adipogenic stages of adipocyte differentiation as well as astrocytes and microglia using RT-PCR (Figure 2.6). In parallel, we confirmed successful ablation of Nucb2 from all analyzed tissues obtained from 12-week old Nucb2 -/- mice.

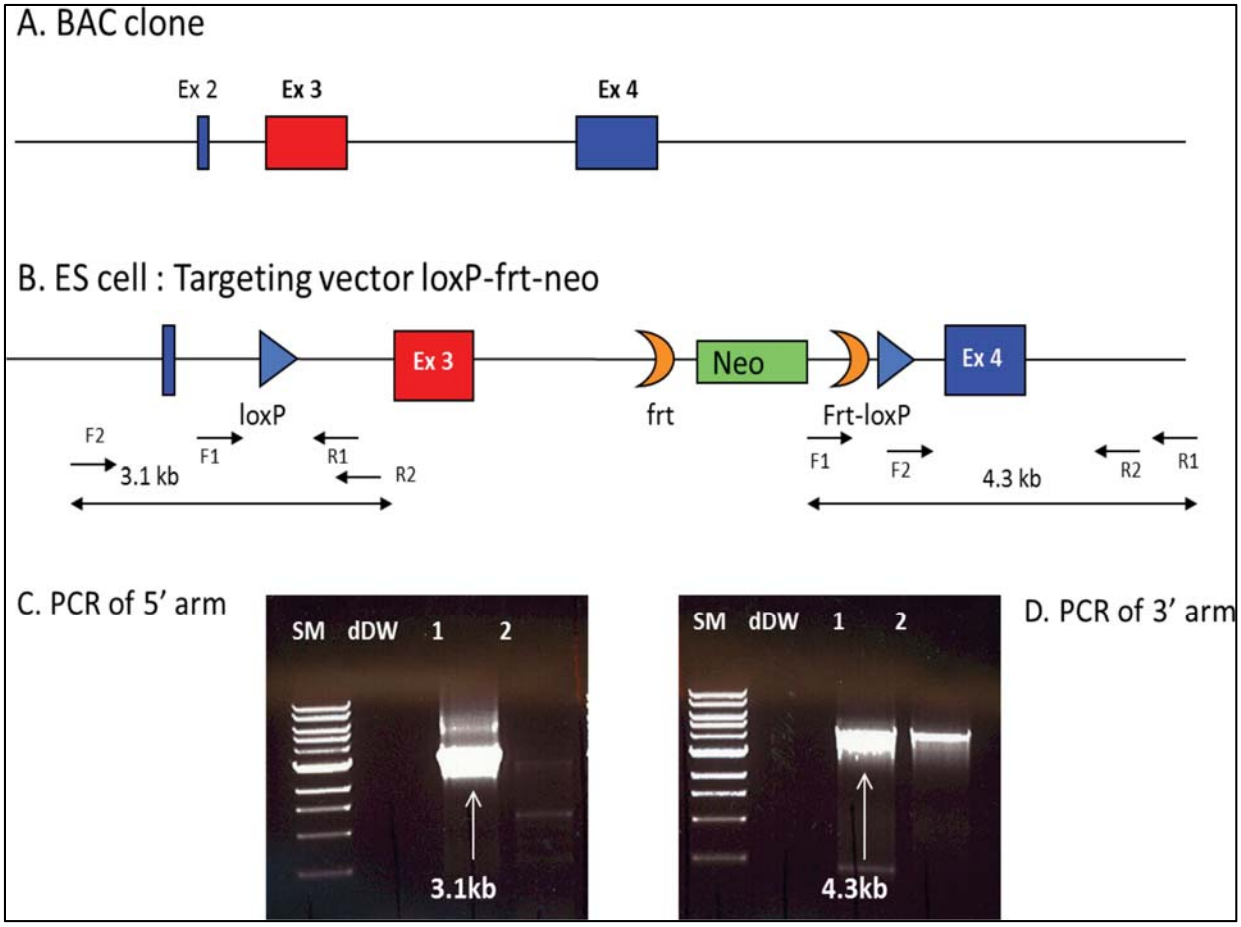


Figure 2.3: *Nucb2* -/- vector construct and embryonic stem cell PCR screens

Vector design depicting the genomic region surrounding exon 3 with the *Neo* construct. (A) Original BAC clone of wild type allele genomic region. (B) *Nucb2* knockout strategy with PL253-*loxP*-frt-neo cassette flanking exon 3. *Frt* is the flippase recognition target and Neo is removed using Flp recombinase once screened for neomycin resistant strains cells. The *Nucb2 loxP* flanked allele is then removed using a ^{Cre}-mediated recombinase. (C) PCR product of the *loxP* flanked allele. Primers were designed for the 5' *loxP* site with a 3.1kb allele confirming insertion of the *loxP* allele. Primers were also designed for the 3' *loxP* site with a 4.3kb allele size (done with help by Dr. Bolormaa Vandanmagsar).

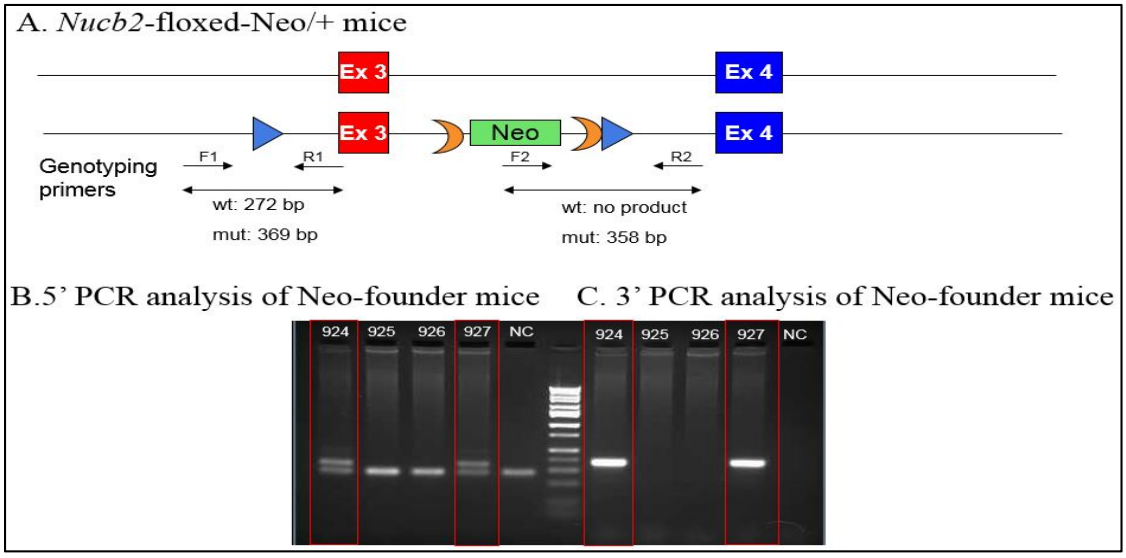


Figure 2.4: *Nucb2* floxed-neo/+ founder mouse and PCR genotyping screen

Genomic targeting diagram depicting region surrounding exon 3 with the *Neo* construct shown in the diagram. (A) *Nucb2* knockout strategy with PL253-*loxP*-frt-neo cassette flanking exon 3. *Frt* is the flippase recognition target and Neo is removed using Flp recombinase once screened for neomycin resistant cells. (B) 5' PCR product of the *Nucb2*-floxed Neo/+ founder mouse (C) 3' PCR product of the *Nucb2*-floxed Neo/+ founder mouse. Red boxes confirm the correct genotyping of our *Nucb2*-neo/+ founder mice.

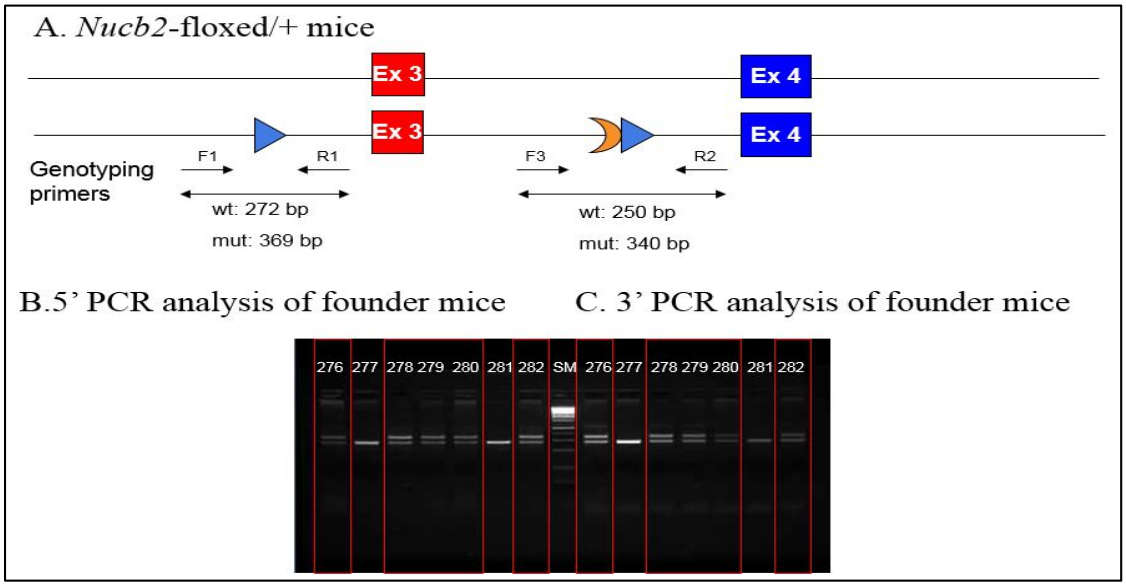


Figure 2.5: *Nucb2*-floxed/+ founder mouse and PCR genotyping screen

Genomic targeting diagram depicting region surrounding exon 3 with the *Neo* construct removed shown in the diagram. (A) *Nucb2* knockout strategy with PL253-*loxP* flanking exon 3. Neo is removed using Flp recombinase once screened for neomycin resistant cells. (B) 5' PCR product of the *Nucb2*-floxed founder mouse (C) 3' PCR product of the *Nucb2*-floxed founder mouse. Red boxes confirm the correct genotyping of our *Nucb2*-floxed+/- founder mice.

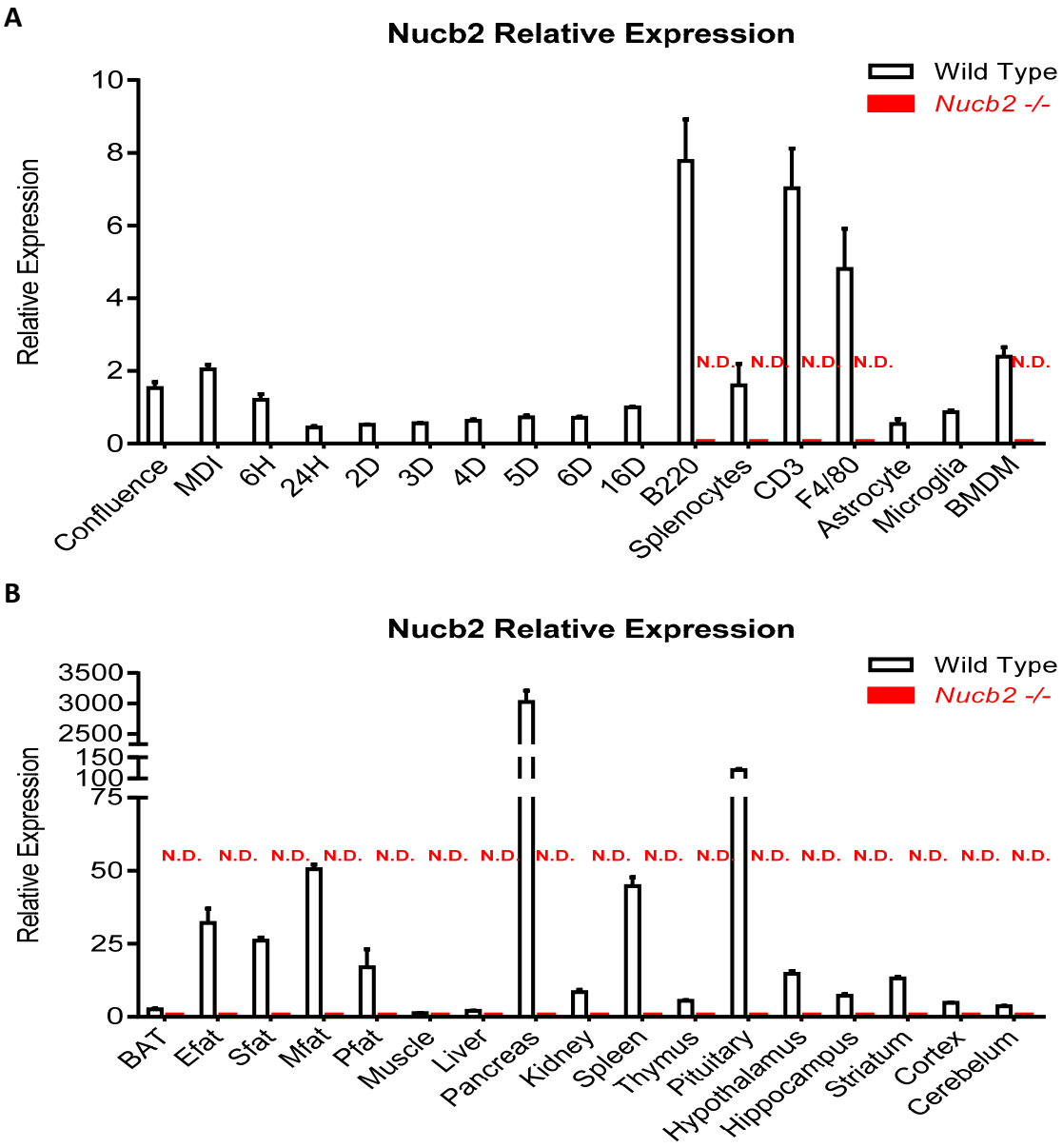


Figure 2.6: *Nucb2* is ubiquitously expressed in many cell types and tissue

Relative *Nucb2* gene expression (mRNA) levels were measured by quantitative RT-PCR. Values are expressed as relative means ± S.E.M. (n=4 per group). (A) Relative *Nucb2* expression in different cell types. There is a high expression of *Nucb2* in B220 B cells, CD3 T cells and F4/80 macrophage positive cells. (B) Relative *Nucb2* expression in different tissues. There is a high *Nucb2* expression level in metabolic tissues (adipose tissues, pancreas and pituitary) as well as in whole spleen.

Together, these data show the correct vector targeting of *Nucb2* gene and that our knockout mice are in fact void of the *Nucb2* gene. This is evident by our inability to detect *Nucb2* gene expression in all the tissues studied in our *Nucb2-/-* mice (Figure 2.6).

2.3.2 Serum nesfatin-1/*Nucb2* expression levels are unchanged and have no effect on food intake and hypothalamic AgRP levels

Nesfatin-1 has been described as a potent regulator of food intake. Oh et al (2006) found that i.c.v injections of nesfatin-1 decrease food intake by regulating melanocortin signaling in the hypothalamus [14]. To determine the physiological response to the ablation of the *Nucb2* gene, we measured food intake as well as hypothalamic agouti-related peptide/protein (*AgRP*) levels, as AgRP is a well characterized orexigenic peptide.

Surprisingly, there were no differences in serum nesfatin-1 levels (Figure 2.7) between fed and fasted wild type mice, and fasted *Nucb2-/-* mice (WT fed, WT fasted and *Nucb2* -/- fasted; 14.39 ± 10.06 ng/ml, 17.36 ± 7.97 ng/ml and 14.41 ± 10.26 ng/ml, respectively, p=0.3 and p=0.8). Wild type and Nucb2 -/- mice fed a HFD also showed no difference in serum nesfatin-1 levels (WT: 3.62 ± 0.38 ng/ml, and Nucb2 -/- 3.21 ± 0.09 ng/ml, respectively, p=0.4) (Figure 2.7). We also ran parallel ELISAs for circulating levels of Nucb2 in the same groups of animals, and similarly, saw no significant differences among any groups. There were no differences in *Nucb2* levels between fed or fasted WT mice, nor fasted *Nucb2* -/- mice (WT fed, WT fasted and *Nucb2* -/- fasted; 665.8 ± 37.9 pg/ml, 598.5 ± 30.7 pg/ml and 578.5 ± 62.3 pg/ml, respectively, p=0.8 and p=0.9). Under high fat conditions, we also saw no difference in *Nucb2* levels between wild type and *Nucb2* -/- mice (710 ± 108.6 pg/ml and 610.4 ± 61.06 pg/ml, respectively, p=0.4) (Figure 2.7).

To further validate the specificity of nesfatin-1 as a secreted peptide derived from Nucb2, we added known amounts of recombinant nesfatin-1, -2 and -3 at concentration of 100 nM and tested the various commercial kits that have been reported in the literature. We did not see nesfatin-1 or *Nucb2* expression in control sample diluent with no serum but spiked with 100 nM nesfatin-1, -2, and -3 recombinant protein (nesfatin-1 levels: 1.59 ± 0.12) (Nucb2 levels: 43.74 ± 5.6 pg/ml) (Figure 2.7). After further examination using recombinant protein as control, we concluded that the ELISA kits are not specific. This is seen clearly between the negative blank control and positive high dose (100 nM) of nesfatin-1, nesfatin-2 and nesfatin-3 spike control (Figure 2.7). In addition, the commercial ELISA kits detected spurious levels of nesfatin-1 in mice that lack the Nucb2 gene. These results question the existence of nesfatin-1 as physiologically relevant peptide that derived from Nucb2.

We next examined the effect of *Nucb2* on food intake and its effects of hypothalamic AgRP responses. Food intake was measured daily over 3 days and averaged. There were no significant differences in food intake between WT mice and *Nucb2* -/- mice fed a chow, ad libitum diet (3.54 ± 0.13 g and 3.65 ± 0.16 g respectively, p=0.6) (Figure 2.8). In concordance with chow diet, there were also no differences between groups fed a HFD (2.8 ± 0.1 g and 2.65 ± 0.15 g respectively, p=0.4) (Figure 2.8).

Corroborating these findings, we saw no differences in hypothalamic AgRP levels between WT and *Nucb2*-/- mice (WT and *Nucb2-/-*: 1.0 ± 0.1 and 1.07 ± 0.15 ΔΔCt fold change, respectively, p=0.7) (Figure 2.8) on a HFD. As expected, AgRP levels significantly increased after a 24 hour fast in both WT and Nucb2 -/- mice (2.51 ± 0.23 and 2.62 ± 0.4 ΔΔCt fold change in wild type and *Nucb2* -/- respectively, p=0.6) (Figure 2.8). Unlike previous reports of nesfatin-1 as a strong regulator of food intake, this data shows that *Nucb2* is not a physiologically important regulator of food intake.

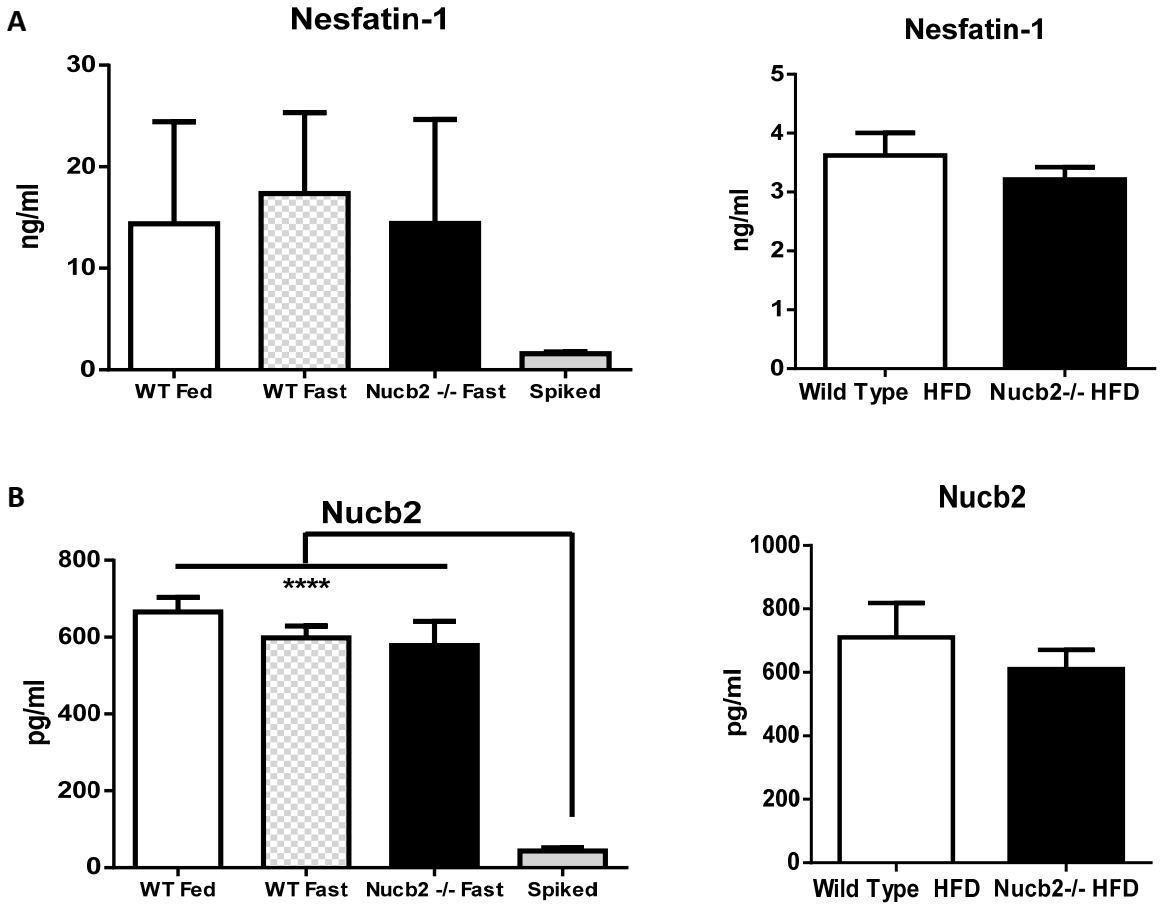


Figure 2.7: *Nucb2* ablation does not alter detected circulating levels of nesfatin-1 or *Nucb2*

(A) There are no differences in Nesfatin-1 and *NUCB2* levels between wild type fed and fasted, as well as fasted *Nucb2 -/-* mice (n=6/group). When the sample diluent was spiked with high levels (100 nM) of recombinant nesfatin-1, nesfatin-2 and nesfatin-3, there was no significant difference in detected levels as compared to the blank. (B) 10 month old, male mice on a high fat diet also had no differences in either nesfatin-1 or *Nucb2* levels between wild type HFD and *Nucb2 -/-* HFD (n=5/group).

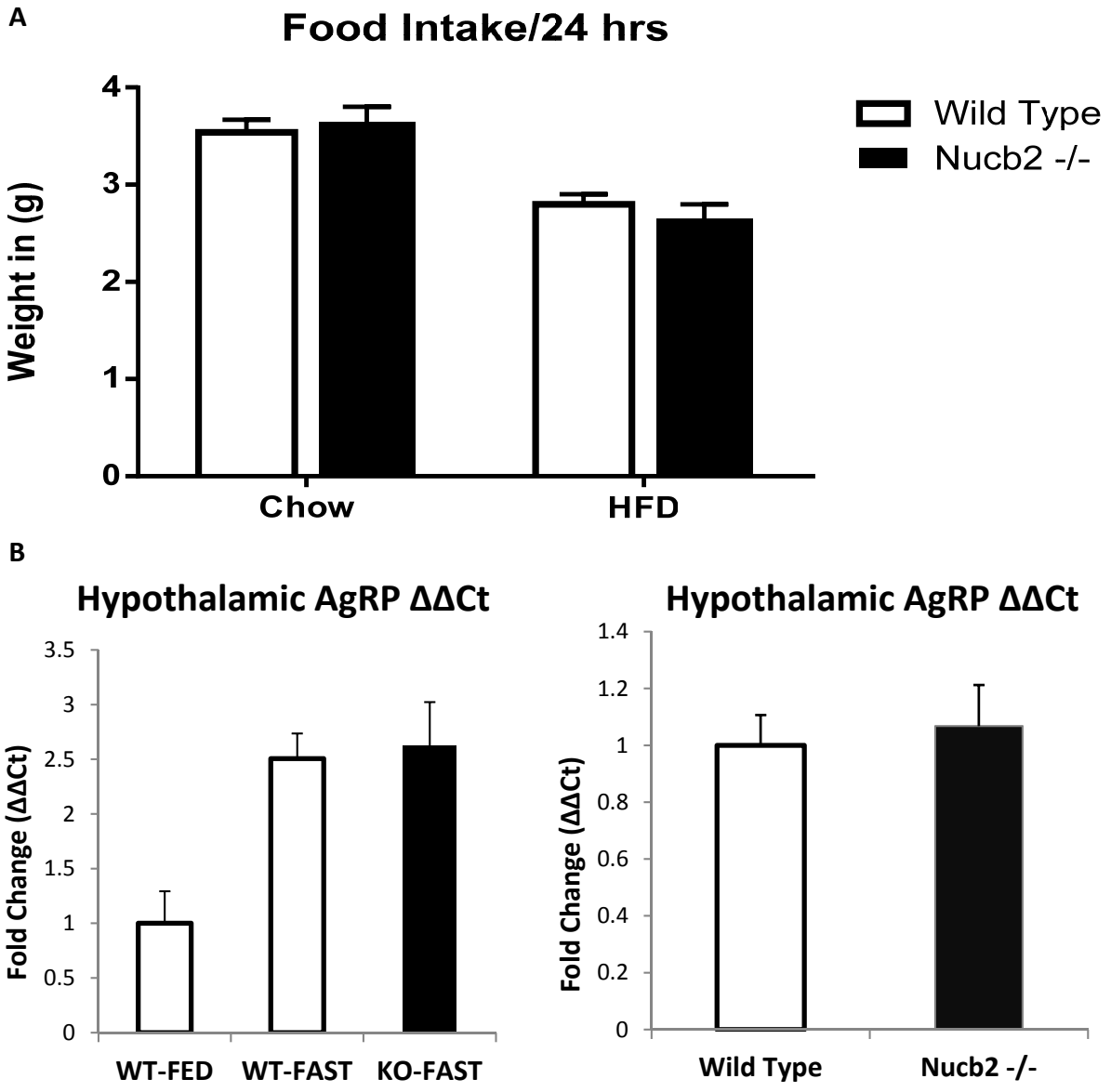


Figure 2.8: *Nucb2* ablation does not alter food intake

Food intake was measured by mass over 72 hours every 12 hours and represented over a 24 hour average (chow, n=8/group HFD, n=5-8/group). (A) There are no differences in cumulative ad libitum food intake over 24 hours between 14 week old chow fed WT and Nucb2 -/- mice, nor between 10 month old HFD fed WT and Nucb2 -/- mice. (B) As expected, fasting significantly increases hypothalamic *AgRP* levels in both WT and *Nucb2 -/- mice (*n=6/group). On a high fat diet, there are also no differences in hypothalamic *AgRP* expression (n=5/group).

These data demonstrates that unlike previous reports [14], *Nucb2* does not impact feeding behavior in both chow fed and fasted mice and HFD fed mice, as well as hypothalamic *AgRP* levels. We also measured levels of *Nucb2* and Nesfatin-1 in periphery. However, our ELISA Results showed no differences between the mice groups, as well as no differences between the

blank samples and the sample diluent spiked with Nesfatin-1, Nesfatin-2 and Nesfatin-3 recombinant protein indicating that this commercial assay is not reliable.

2.3.3 *Nucb2* has no effect on body weight and body composition

We next examined whether *Nucb2* influences body weight and body composition in animals fed both normal chow and HFD. There were no significant changes in body weight or body composition, as assessed by proton NMR, between the WT and *Nucb2*-/- littermate mice in both chow and high fat fed conditions. This was seen at various time points sampled throughout the lifespan of the mice. Young, 14 week old WT mice fed a chow diet weighed 28.65 ± 0.55 (n=8) and Nucb2 -/- mice weighed 30.06 ± 0.82g (n=7), p=0.2 (Figure 2.9). During ad libitum high fat feeding, 12 week old wild type mice weighed 27.02± 0.6 (n=11) and the *Nucb2*-/- mice weighed 28.51 ± 0.63g (n=7), p=0.1 (Figure 2.9). Using proton NMR to measure differences in body composition, we found no statistical differences in fat mass between wild type and *Nucb2* -/- mice. On the chow diet, wild type mice had 2.05± 0.14g of fat, whereas *Nucb2* -/- mice had 1.86 ± 0.44g of fat, p=04. On the high fat diet, wild type mice had 4.8 ± 0.4g of fat, whereas *Nucb2* -/- mice had 5.65 ± 0.5g of fat, p=0.2. We therefore conclude Nucb2 does not play a role in regulating body weight or body composition in either chow or HFD conditions.

Moreover, to determine if *Nucb2* had an effect on body weight and adiposity in the long term (at an older age), we chronically fed high fat diet to WT and *Nucb2-/-* mice for 10 months. *Nucb2* $^{-/-}$ mice showed similar body weight and fat mass as the WT controls (WT HFD 52.25 ± 1.306g (n=8), *Nucb2* -/- HFD mice weight 52.60 ± 1.12g, p=0.9 (n=5) (Figure 2.9). In addition to having no differences in body weight, *Nucb2* -/- mice also showed no differences in body mass composition as compared to control (Figure 2.9). Visceral fat mass (1.41 ± 0.15 g and 1.34 ± 0.08 g, p=0.7), inguinal fat mass (2.49 ± 0.14 g and 2.1 ± 0.13 g, p=0.1) and perirenal fat mass (1.21 ± 0.06 g and 1.19 ± 0.06 g, p=0.8) were no different between WT control and *Nucb2* -/- mice. This data suggests *Nucb2* is not involved in the maintenance of body weight or body composition in either chow or high fat conditions.

To summarize, *Nucb2* is not an important regulator of body weight nor body composition during both chow fed and HFD conditions. We observe no differences in body weight nor body composition between the wild type control groups when compared to littermate *Nucb2*-/- mice.

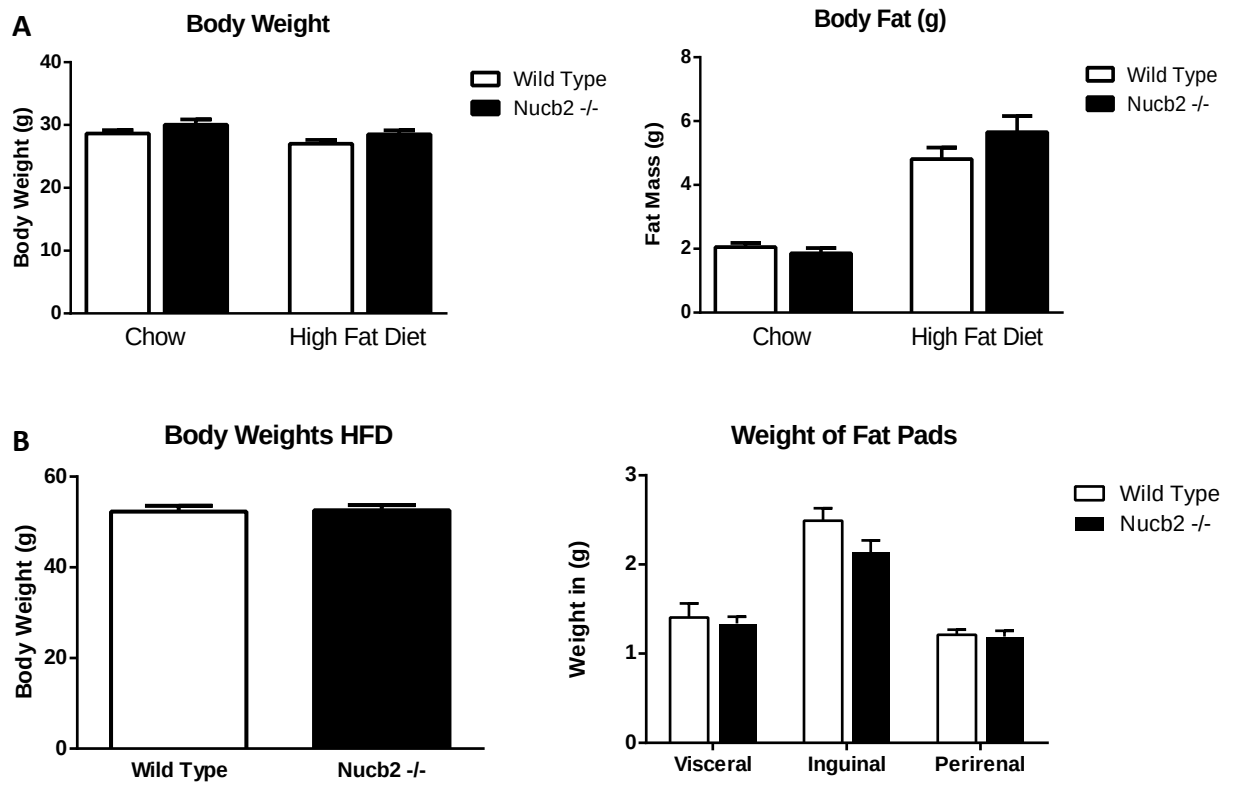


Figure 2.9: *Nucb2* ablation has no effect on body weight and body composition

(A) Body weight and body fat in grams of 14 week old chow-fed mice (WT n=8, KO n=7) and 12 week old HFD-fed mice (WT n=11, KO n=7). (B) Body weight of 10 month old HFD-fed mice and different adipose tissue weights (WT n=8, KO n=5).

2.3.4 *Nucb2* has no impact on adipose tissue morphology, hypertrophy and hyperplasia

More than overall adipose tissue size (adiposity), both individual adipocyte size itself and micro-environment are vital in normal glucose homeostasis and in the maintenance of glucose disposal [148]. Further, an adipocyte's inability to expand and store lipid in obese conditions is associated with insulin resistance due to ectopic fat stores and lipid-spillover in liver and muscle [149]. We thus sought to determine if knockout of *Nucb2* gene induced changes in adipocyte size, or in the morphology of adipose tissue. Histological appearances of the white adipose tissue are shown in (Figure 2.10). Using hematoxylin and eosin (H&E) staining in a Texas red filter, we observed no difference in adipocyte morphology. Further quantification showed no difference in average adipocyte size between the wild type (6372 ± 200.3 um^2) and *Nucb2* $^{-/-}$ (5942 ± 204.0 um^2) mice, p=0.1.

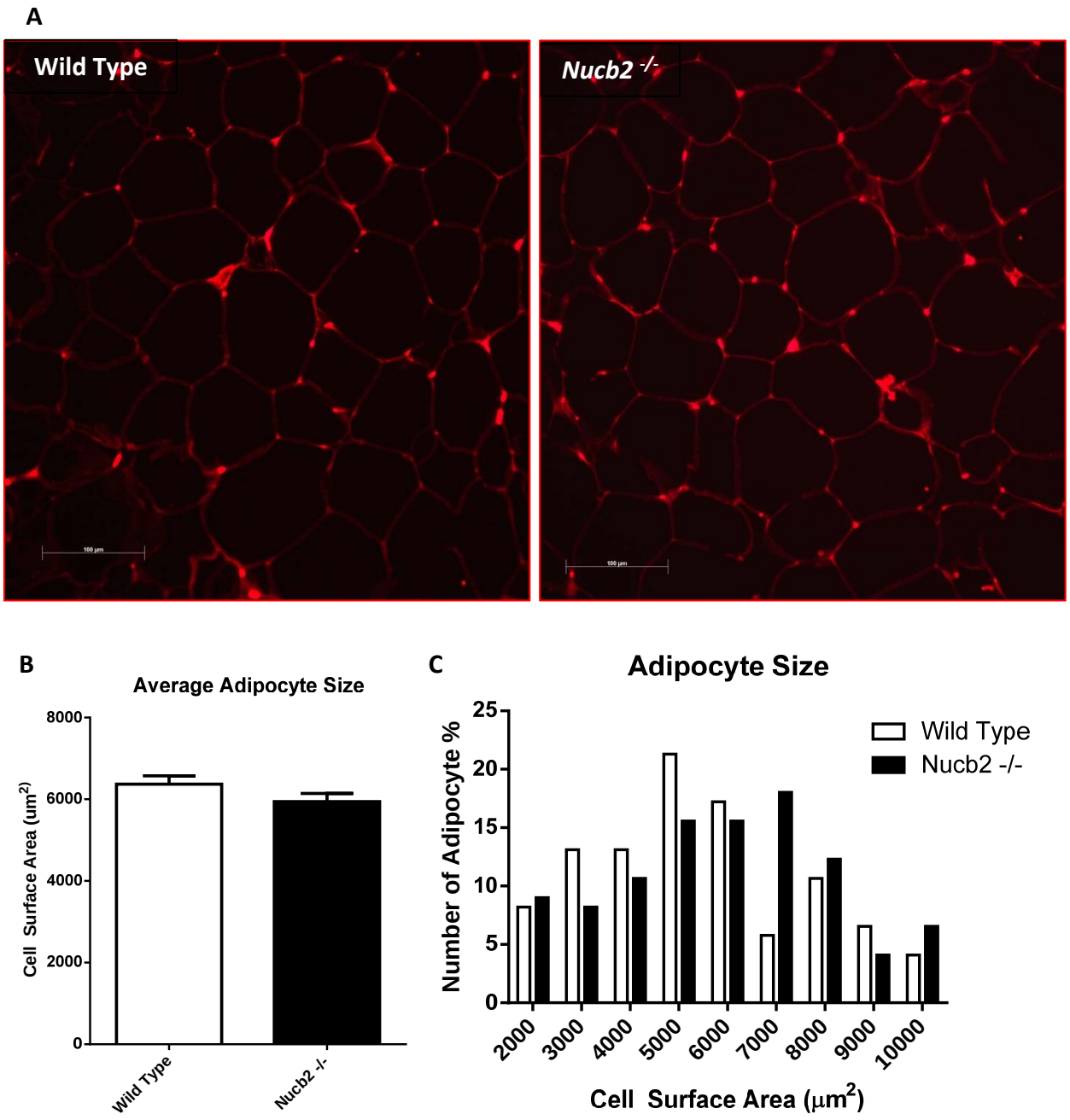


Figure 2.10: *Nucb2* ablation does not impact adipocyte morphology and adipocyte size

(A) Adipose tissue stained with H&E from 10 month old wild type and *Nucb2-/- mice* fed an ad libitum high fat diet and photographed at x20 magnification. Scale bar = 100um. (B) The average size of adipocyte was measured by using Image J and averaging over 180 different cells from 4 different mice/strain. (C) Size distribution histogram from 4 mice/group.

Overall, these data demonstrate that there is no difference in adipose tissue morphology, or adipocyte size between the wild type controls and *Nucb2 -/-* mice at 10 months obesity induced high fat diet.

2.3.5 Serum metabolites and cytokines are mostly unaffected in *Nucb2-/-* mice

Next, we wanted to determine if *Nucb2* has an effect on circulating peripheral metabolites under different metabolic conditions. Yadav et al. (2013) showed that different circulating cytokines, as well as adiponectin, which is inversely correlated with adipose tissue size, and leptin, which is directly correlated to adipose tissue size, can potentially regulate insulin resistance [150]. Analyses of metabolites and cytokines were performed for in different experiments. The first experiment included 3 groups of 6 month old, male mice; wild type mice fed an ad libitum chow diet or fasted for 24 hours, and *Nucb2-/-* 24 hour fasted mice (n=6/group). The second experiment was performed on both wild type and *Nucb2-/-* HFD-fed 10 month old male mice (n=5/group).

Since adiponectin has been shown to be inversely correlated with adipose tissue size, and to mediate an insulin-sensitizing effect by binding to its receptors AdipoR1 and AdipoR2, we wanted to see if ablation of *Nucb2* affected adiponectin levels. First, we identified a trend towards increasing adiponectin levels in fasted mice; however there was no difference in adiponectin levels between wild type and *Nucb2*-/- mice in a fasted state (149 ± 6.1 and 150 ± 6.9, respectively, p=0.9). Under high fat conditions, we also saw no difference in adiponectin levels between wild type and *Nucb2*-/- mice (151.1 ± 1.38 and 159.1 ± 4.59, respectively) (Figure 2.12).

In addition to a lack of effect of *Nucb2* knockout on adiponectin levels, there were no differences in circulating leptin levels between wild type and *Nucb2* -/- mice both under fasting conditions and HFD conditions. There was a significant drop in leptin levels after fasting (WT, fed vs fasted: 2583 ± 419 pg/ml and 1229 ± 370 pg/ml respectively, p=0.04) and this was also seen in *Nucb2* -/- mice (*Nucb2*-/-, fed vs fasted: 2583 ± 419 pg/ml and 587.5 ± 450 pg/ml respectively, p=0.001). We observed no difference in leptin levels between wild type and *Nucb2* -/- fasted mice (p=0.1). Furthermore, there were no differences in leptin levels between WT and Nucb2 -/- mice fed a high fat diet (WT and Nucb 2-/-: 5068 ± 214 pg/ml and 5064 ± 286 pg/ml, respectively, p=0.99) (Figure 2.12).

In addition to looking at circulating adipokines and metabolites, we also measured how *Nucb2* affects systemic peripheral cytokines. Changes in circulating cytokine levels have been associated with many diseases; however, most cytokines circulate in peripheral blood in very low concentrations and are very difficult to detect under sterile conditions. IL-18, on the other hand, has been shown to have a constitutive pool and is thus easily measurable in serum. Furthermore,

IL-18 has been reported to be an important insulin sensitizer [151]. We thus measured serum levels of IL-18.

There was a trend towards a fasting-induced increase in IL-18 levels in wild type mice (WT fed vs fasted: 622.5 ± 20.2 pg/ml and 731.3 ± 60.9 pg/ml respectively p=0.1), however, not significant. We observed the opposite in Nucb2 -/- mice, where fasting induced a significant decrease in IL -18 levels as compared to fasted wild type mice (WT vs *Nucb2*-/- fasted: 731.3 ± 60.9 pg/ml and 505.8 ± 42.7 pg/ml respectively, p=0.01) and also a decrease IL-18 in *Nucb2*-/- mice compared to WT fed mice (p=0.03) (Figure 2.12). Furthermore, there was no significant difference in IL-18 levels in wild type versus Nucb2 -/- mice fed a high fat diet (HFD-WT and HFD-Nucb 2-/-: 741.7 ± 66.9 pg/ml and 597.2 ± 37.8 pg/ml, respectively p=0.1) (Figure 2.12), despite a decreasing trend in the *Nucb2* -/- HFD mice.

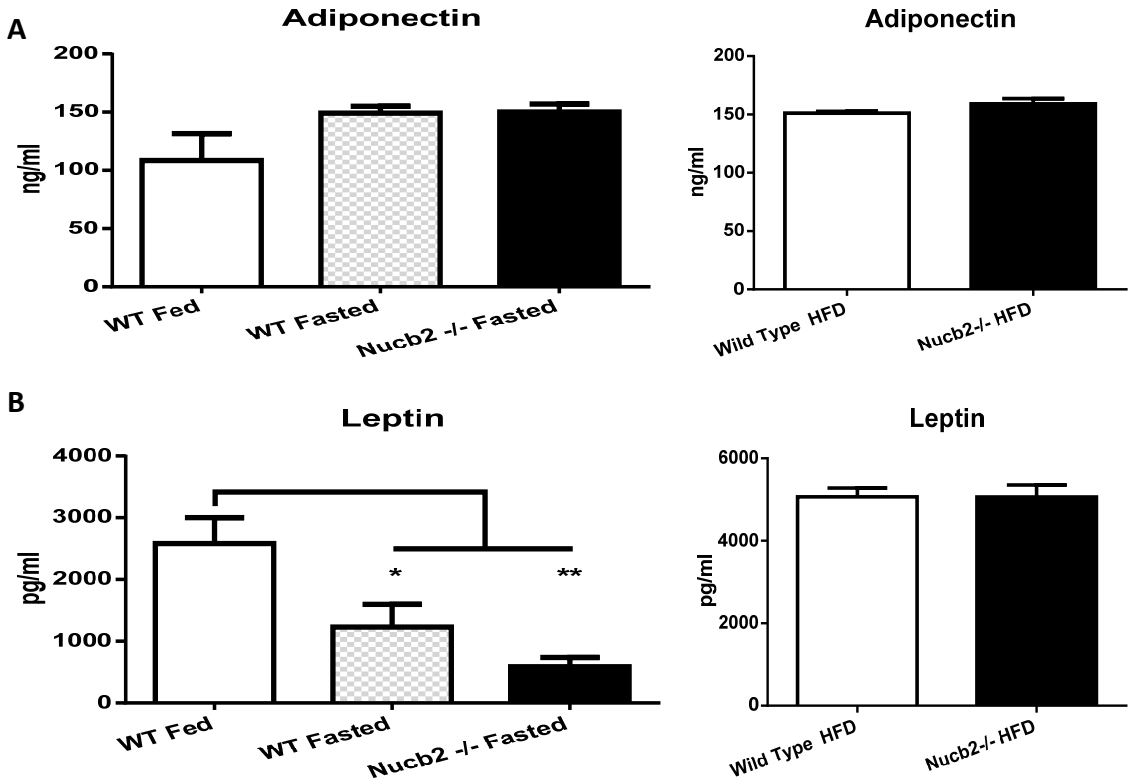


Figure 2.11: *Nucb2* ablation does not affect circulating adipokines in HFD fed mice

(A) Adiponectin Levels are no different between wild type chow fed and fasted mice and Nucb2-/- fasted mice (n=6/group), and between wild type and Nucb2 -/- HFD mice (n=5/group). (B) There is a decrease in Leptin levels during fasting states in both wild type and Nucb2-/-, however, there is no significant difference between wild type and Nucb2 -/- (n=6/group). There is an increase in Leptin levels of mice on a HFD, however, there is no difference between wild type and Nucb2-/- (n=5/group).

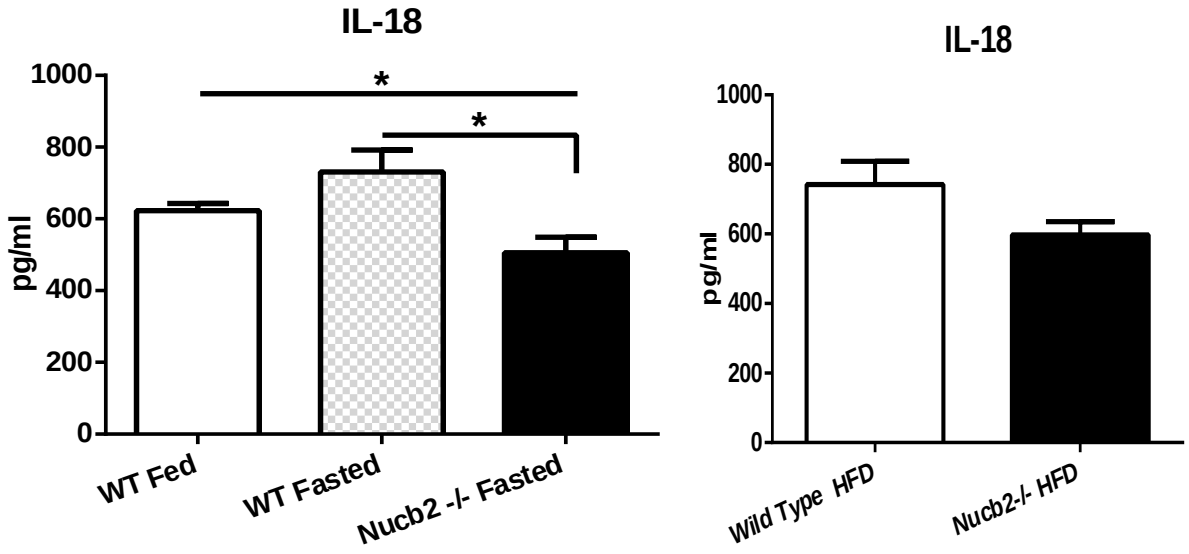


Figure 2.12: *Nucb2* ablation does not affect circulating IL-18 in HFD fed mice

There is no difference between chow fed and fasted wild type mice, however, there is a slight decrease between wild type fasted mice and *Nucb2-/-* fasted mice (n=6/group). There is a decreasing trend in the *Nucb2-/-* HFD mice when compared to wild type, however this is not significant (n=5/group).

Overall, there does not seem to be a difference in levels of serum metabolic hormones between the wild type mice and the *Nucb2 -/-* mice in different metabolic states. On the other hand, *Nucb2-/-* mice have a significantly lower IL-18 expression in fasted mice, and a trend of lower IL-18 levels in HFD *Nucb2-/-* mice.

2.3.6 *Nucb2* does not regulate insulin signaling or glucose homeostasis in ad-libitum fed chow mice

To test if *Nucb2* is important in regulating metabolism and whole-body insulin action, we performed euglycemic-hyperinsulinemic clamps on 14 week old mice fed ad libitum chow diets. As described above, wild type (n=8) and *Nucb2 -/-* (n=7) mice had no significant differences in body weight (28.65g ± 0.55g and 30.06g ± 0.82g respectively, p=0.2). Furthermore, there was no difference in the fat mass between wild type and *Nucb2 -/-* mice (2.05 ± 0.14 and 1.86 ± 0.27 respectively, p=0.38).

Compared to wild type mice, *Nucb2 -/-* mice showed no differences in whole-body insulin sensitivity. Indeed, compared to wild type mice, *Nucb2 -/-* mice required similar glucose infusion rate to maintain euglycemia during the entirety of the hyperinsulinemic-euglycemic clamp (Figure 2.13). There were also no significant differences between wild type and *Nucb2 -/-* mice in peripheral glucose uptake (42.6 ± 2.6 and 46.6 ± 3.9 respectively, p=0.4) as well as no effects on endogenous hepatic glucose production (EGP) in either the basal (15.0 ± 1.3 and 14.9 ± 0.9

respectively, p=0.96) or the clamped state (1.5 ± 1.8 and 0.5 ± 1.0 respectively, p=0.62) (Figure 2.13). The sharp decrease in EGP through the clamp study shows high insulin sensitivity in the liver in both mouse models.

There was also no dysfunction in pancreatic insulin secretion. Wild type and *Nucb2* -/- mice showed no difference in insulin production in both basal (5.2 ± 0.6 and 4.4 ± 1.7 respectively, p=0.4) and clamped states (34.6 ± 1.9 and 35.6 ± 2.2 respectively, p=0.7). In line with these data, adipocyte function did not appear to be affected by *Nucb2-/-* in chow-feeding, as reflected by no difference in levels of insulin-stimulated plasma free fatty acids between wild type and *Nucb2* -/- mice (basal FFA: 1.06 ± 0.06 mEq/l and 1.11 ± 0.12, respectively, p=0.8), and clamped FFA: 0.34 ± 0.06 and 0.38 ± 0.09, respectively, p=0.7). This result is reflected by a 66% insulin-stimulated suppression of plasma fatty acids (Figure 2.14).

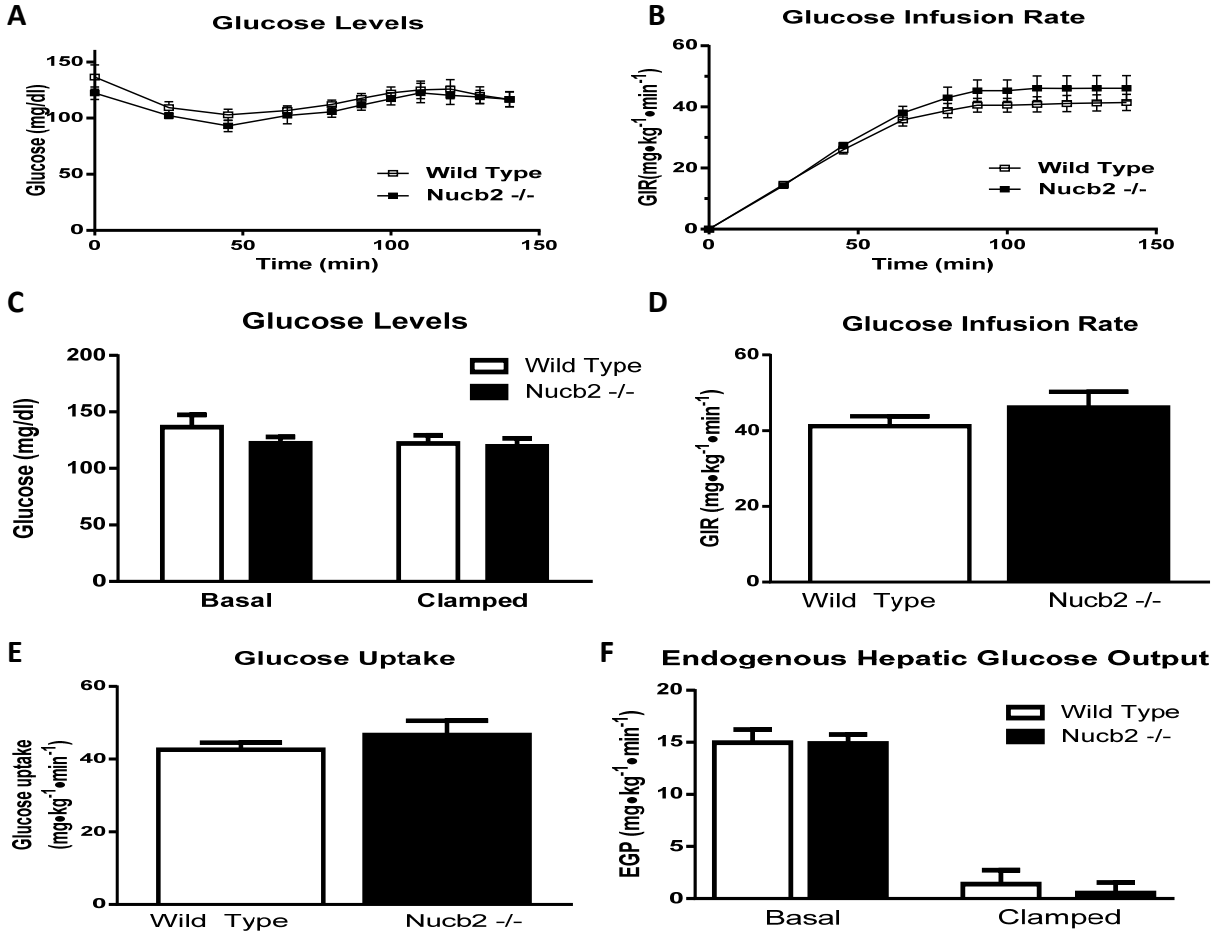


Figure 2.13: *Nucb2* does not affect whole body glucose homeostasis in lean chow fed mice

(A) Time-course of plasma glucose levels (B) Glucose Infusion Rate (C) Basal and Clamped plasma glucose levels (D) End-point glucose infusion rate (E) Systemic glucose uptake during the steady-state period (final 40 min) of the clamp (F) and Basal and insulin-stimulated clamped hepatic endogenous glucose production. Male 14 week old chow fed mice and data are represented as mean ± SEM (wild type, n=8 and *Nucb2 -/-,* n=7)

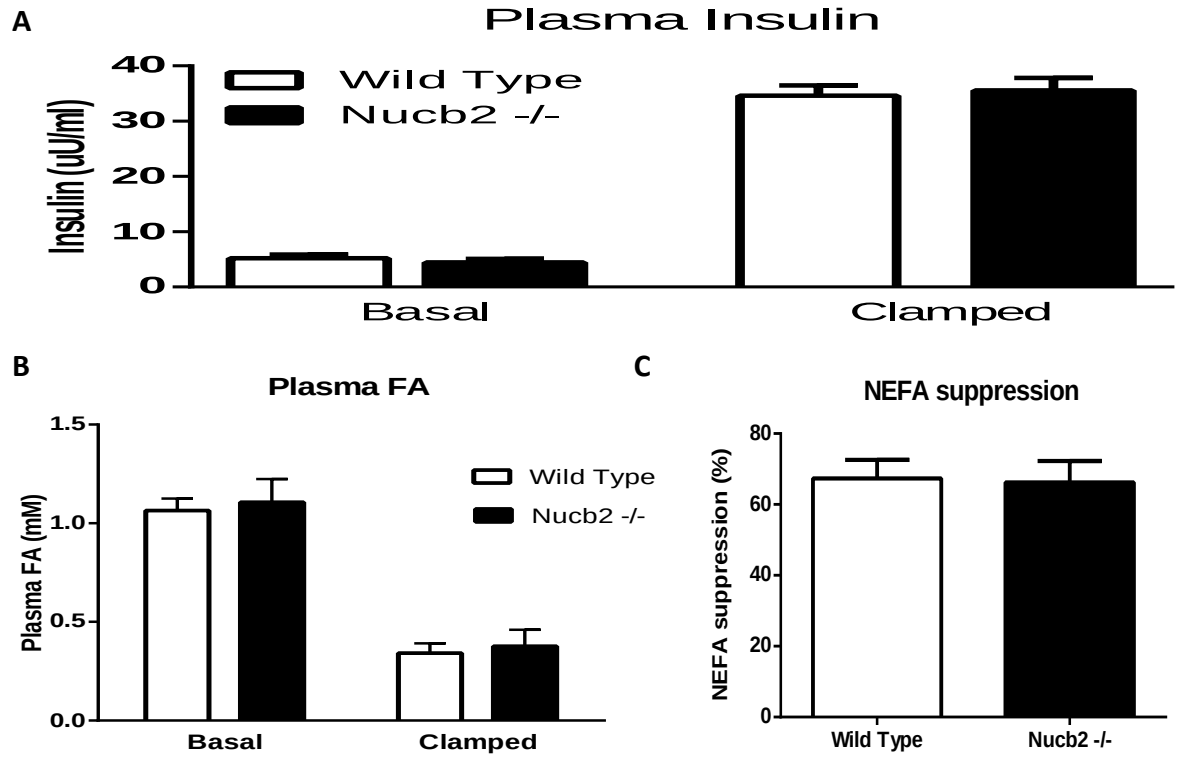


Figure 2.14: *Nucb2* does not affect plasma insulin or plasma fatty acids

(A) Basal and clamped plasma insulin levels (B) Basal and insulin-stimulated clamped plasma free fatty acid levels (C) NEFA (Non-esterified Fatty Acids) Plasma fatty acid suppression. Data are represented as mean ± SEM (wild type, n=8 and *Nucb2 -/-,* n=7)

These data suggest that *Nucb2* is not required for insulin signaling and overall glucose homeostasis under normal, metabolically healthy chow fed conditions, as both *Nucb2-/-* mice and wild type mice required a similar amount of glucose infusion to maintain euglycemia. Moreover, there are no differences in plasma insulin, or plasma fatty acids between wild type and *Nucb2-/-* mice.

2.3.7 *Nucb2* is necessary for proper insulin signaling and regulation of glucose homeostasis in high fat challenged mice

To determine if *Nucb2* is important in the regulation of whole-body insulin action during obesity-induced insulin resistance (HFD), we next performed hyperinsulinemic-euglycemic clamps on WT and *Nucb2-/-* mice that were fed a 60% Kcal high fat diet for 12 weeks. Basal characteristics (body weight and body fat mass) were not different between wild type and *Nucb2* -/- mice (body weight: 27.02 ± 0.6 g and 28.51 ± 063 g, respectively, p=0.1 and body fat: 4.81 ± 0.36 g and 5.7 ± 0.5 g, respectively p=0.2) (Figure 2.15).

In contrast to the results in the chow fed mice, *Nucb2*-/- mice fed a high fat diet displayed a significant whole-body insulin resistance as reflected by an almost 50% decrease in the glucose infusion rate (21.3 ± 2.1 and 11.0 ± 0.9, wild type and *Nucb2* -/- respectively, p=0.002) required to maintain euglycemia during the hyperinsulinemic-euglycemic clamp (Figure 2.16B).

There were no statistically significant differences between wild type and *Nucb2* -/- mice in whole-body glucose uptake (29.25 ± 2.24 and 24.8 ± 2.3 respectively, p=0.2) despite a trend towards lower values in the knock out mice (Figure 2.16E). On the other hand, there was a significant increase in EGP in the *Nucb2* -/- clamped mice (wild type and *Nucb2* -/-: 7.96 ± 1.75 and 13.79 ± 1.71 respectively p=0.04) but not in the basal state (wild type and *Nucb2* -/-: 11.29 ± 0.88 and 10.97 ± 1.11 respectively p=0.8) (Figure 2.16F). This clearly suggests a state of liver insulin resistance in which the liver is unable to shut down glucose production (primarily through glycogenolysis and gluconeogenesis) in the face of increased in insulin levels.

We did not observe any effects of Nucb2 knockout on pancreatic insulin secretion. Wild type and *Nucb2* -/- mice showed no difference in insulin production in both basal (12.41 ± 1.47 and 14.19 ± 1.4 respectively, p=0.4) and clamped states (76.65 ± 6.3 and 75.23 ± 3.5 respectively, p=0.9) (Figure 2.17). Remarkably, however, there seemed to be adipocyte dysfunction in *Nucb2* -/- mice on a high fat diet, as reflected by a significant increase in insulin-stimulated plasma free fatty acids in the *Nucb2* -/- mice compared to wild type mice (basal FFA: 0.83 ± 0.06 mEq/l and 0.69 ± 0.09, respectively, p=0.2), and clamped FFA: 0.4 ± 0.03 and 0.63 ± 0.04, respectively, p=0.0003) (Figure 2.17). This resulted in a 57% FFA suppression in wild type mice, and only a 2% suppression in the *Nucb2* -/- mice under clamped conditions. This suggests adipose tissue insulin resistance, in which the adipose tissue does not shut down triglyceride lipolysis and continues to release nonesterified fatty acids even though there is glucose available as energy in the clamped state.

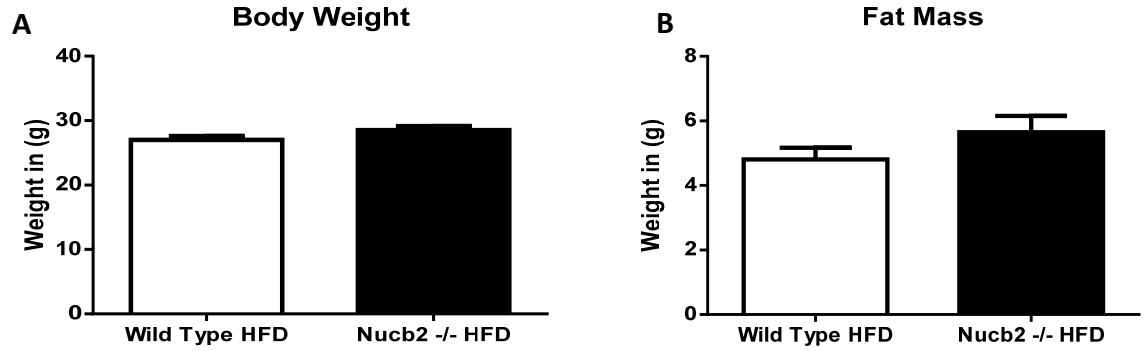


Figure 2.15: *Nucb2* has no effect on body weight and body composition in HFD fed mice

(A) Body weight in grams and (B) fat mass in grams. All data are represented as mean ± SEM (12 week old, male wild type, n=11 and *Nucb2 -/-,* n=8)

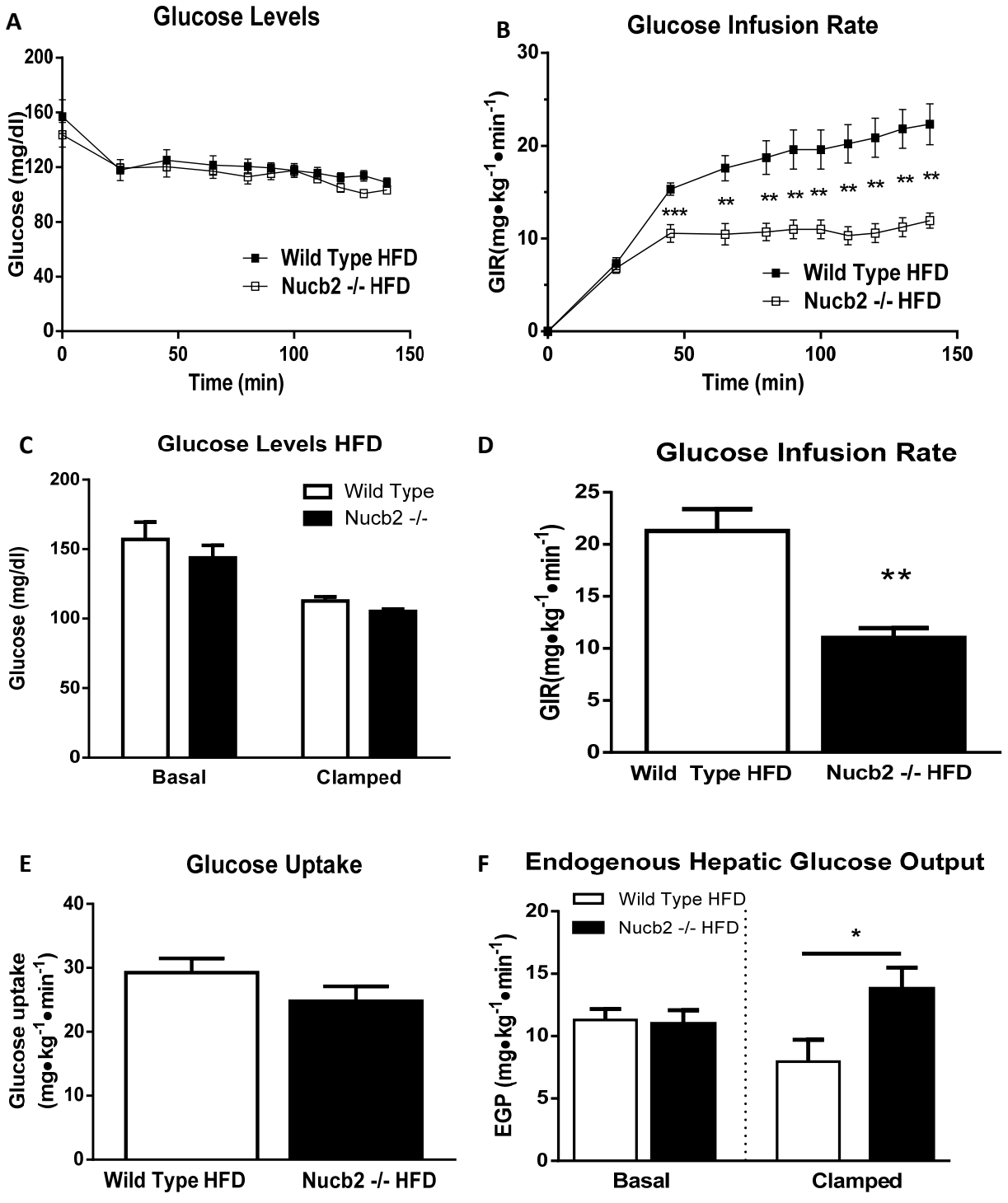


Figure 2.16: *Nucb2* ablation causes systemic and hepatic insulin-resistance in HFD fed mice

(A) Time-course of plasma glucose levels (B) Glucose Infusion Rate (C) Basal and Clamped plasma glucose levels (D) End-point glucose infusion rate (E) Systemic glucose uptake during the steady-state period (final 40 min) of the clamp (F) and Basal and insulin-stimulated clamped endogenous glucose output. Data are represented as mean ± SEM (wild type, n=11 and *Nucb2 -/-,* n=8)

Overall, these data illustrate that ablation of *Nucb2* negatively impacts normal glucose disposal and normal insulin signaling in animals fed a high fat diet. This is clearly seen by the ability of wild type mice to clear infused glucose at a much higher rate than the *Nucb2 -/-* mice. Moreover, the ablation of *Nucb2* significantly alters liver insulin sensitivity, as seen by the knockout animals' inability to reduce hepatic glucose production under insulin-stimulated clamped conditions.

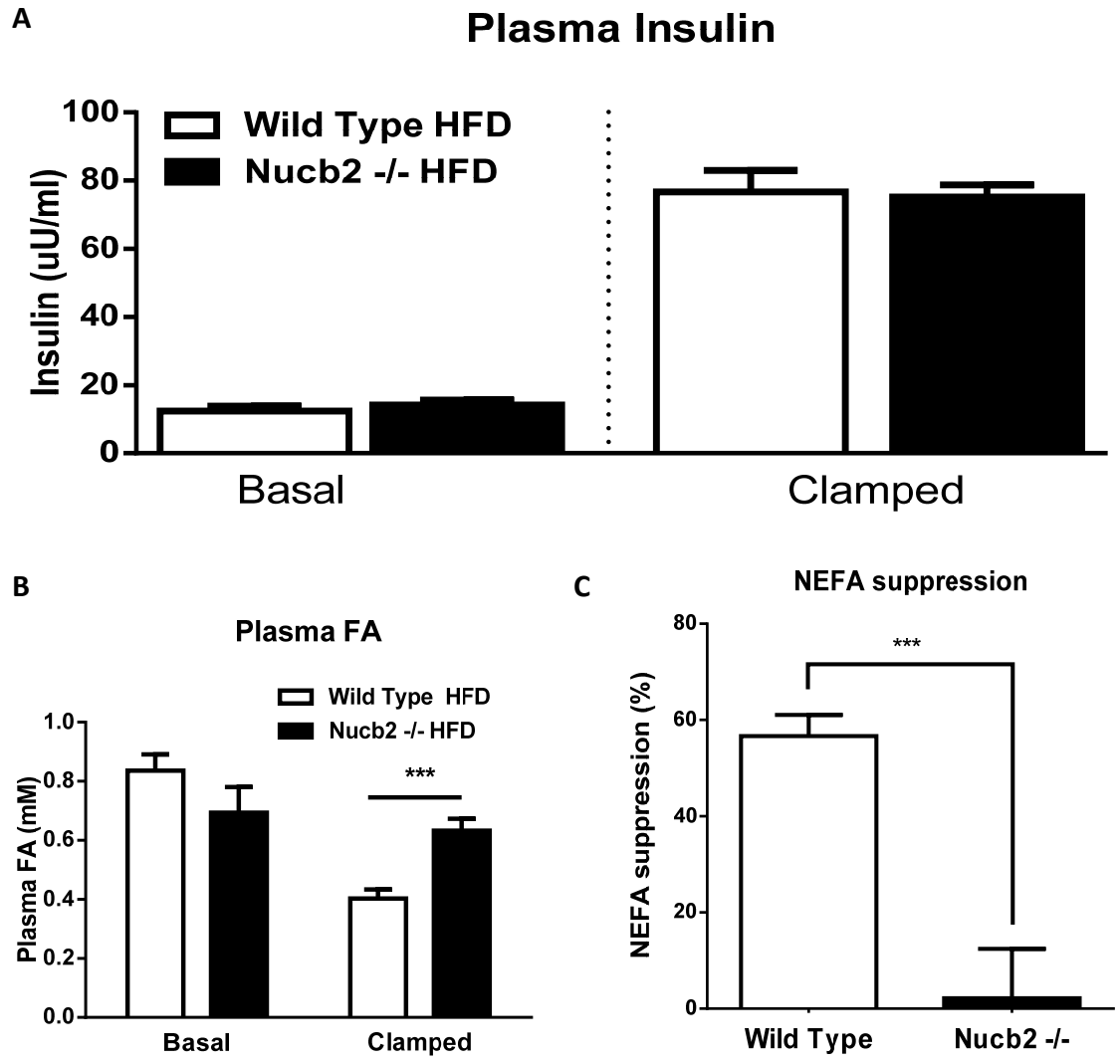


Figure 2.17: *Nucb2* ablation has no effect on insulin secretion but leads to adipocyte insulin resistance

(A) Basal and clamped plasma insulin levels (B) Basal and insulin-stimulated clamped plasma free fatty acid levels (C) Plasma fatty acid suppression. Data are represented as mean ± SEM (wild type, n=11 and *Nucb2 -/-,* n=8)

These data further show that ablation of *Nucb2* does not affect insulin secretion. *Nucb2* -/- mice do, however, show significant adipose tissue insulin resistance as evidenced by their inability to suppress the lipolysis and ultimate secretion of fatty acids into plasma throughout the course of the clamp.

2.4 Discussion

The major findings of these studies are: **1)** While *Nucb2* expression is not regulated by diet or age in the hypothalamus, liver or visceral adipose tissue, it is significantly up-regulated in isolated macrophages and T cells derived from the adipose tissue of HFD-fed mice; **2)** *Nucb2* does not modulate food intake, body weight and body composition; **3)** *Nucb2* is not required for normal glucose homeostasis, insulin secretion and insulin sensitivity in lean, chow fed mice. **4)** *Nucb2* is, however, required for insulin sensitivity and glucose homeostasis during hypercaloric, high fat diet feeding as loss of Nucb2 causes severe insulin-resistance in obesity.

Previous studies have shown nesfatin-1 to be an anorexigenic molecule [14, 152]. Our data, however show that removal of the *Nucb2* gene, and thus endogenous, physiological levels of Nucb2, does not affect food intake, as assessed by both direct measurement of food consumed as well as mRNA expression of AgRP. Together, these findings indicate that *Nucb2* is not an important regulator of feeding and that it does not interact with the neuropeptides NPY and AgRP, themselves accepted regulators of food intake [153-156]. It is plausible that previous studies demonstrating anorexigenic actions implicative of *Nucb2* might see a pharmacological effect of supraphysiological doses of intracerebroventricularly injected nesfatin-1 [14, 23, 129, 140]. It is important to note, however, that previous studies using commercially available antibodies are unable to specifically detect nesfatin-1. Furthermore, nesfatin-1 alone may affect food intake, while *Nucb2* may have pleiotropic effects that are compensated or controlled by nesfatin-2 or nesfatin-3, whose functions remain unknown. To further delineate the importance of these three individual peptides, it would be necessary to inhibit only one or use recombinant proteins to reintroduce each peptide alone and in combinations with one another. In support of our demonstrations of a lack of effect of Nucb2 on ingestive behavior, however, is a recent study showing that *Nucb2/Nesfatin-1* does not change daily food intake or modify mRNA expression levels of orexigenic (NPY, AgRP) or anorexigenic (POMC) proteins [157]. Interestingly, much like our genetic approach, this study used a genetic overexpression model of *Nucb2,* whereas previous studies linking Nucb2/nesfatin-1 to regulation of food intake were all pharmacologic.

The magnitude of reduced glucose infusion during a hyperinsulinemic-euglycemic clamp in *Nucb2* deficient mice on a high fat diet is drastic (50%), clearly indicating a role of Nucb2 in the regulation pathways of insulin sensitivity. Furthermore, the data show that *Nucb2* is important for insulin sensitivity especially in adipose tissue by its inability to suppress lipolysis and

circulating free fatty acids and in liver which becomes unable to shutdown endogenous glucose production during the insulin-stimulated clamp. Together, these data indicate that *Nucb2* is necessary for appropriate insulin signaling and glucose uptake in metabolic tissues in diet-induced obesity and its related insulin impaired insulin resistance.

Consistent with initial cloning of *Nucb2* in B-lymphocyte cell lines, *Nucb2* is highly expressed in different leukocyte subpopulations such as macrophages, T-lymphocytes and plasmacytoid dentritic cells. Furthermore, *Nucb2* expression levels in adipose derived macrophage and T cells are positively regulated by a high fat diet. In line with our observations, other studies have shown that *Nucb2* and nesfatin-1 levels are significantly higher in different depots in high-fat fed mice and reduced under food deprivation [134]. This group further showed that stimulation of subcutaneous adipose tissue explants with cytokines TNF-α and IL-6, insulin and dexamethasone significantly increases intracellular nesfatin-1 levels [134]. Due to the high expression of *Nucb2* in immune cells and an increase in leukocyte *Nucb2* expression following high fat feeding, we hypothesize that *Nucb2* is important in the activation and/or regulation of immune cells.

Our results confirmed the importance of *Nucb2* in regulating local metabolism (occurrence of local insulin resistance) thus probably protecting the tissue from more deleterious damage (cell death for example). It also identifies a dissociation between the physiological regulation of energy metabolism (energy intake and energy expenditure) and the level of insulin resistance. Such observation may remind us that all the anabolic periods of life such as puberty and pregnancy are first characterized by a state of insulin resistance.

In conclusion, ablation of *Nucb2* failed to influence body weight, body composition or food intake under chow or high fat diet conditions, thus suggesting that *Nucb2* has no role in the physiological regulation of food intake or overall energy metabolism. On the other hand, *Nucb2* appears to be an important insulin sensitizer during obesity-induced insulin resistant states, and is required for normal glucose metabolism and glucose homeostasis. This is evident by the derangement in glucose homeostasis in the *Nucb2 -/-* mice. Weisberg et al. (2003) showed that leukocytosis in adipose tissue is directly correlated with adiposity [158] and that increased expression of immune cell proinflammatory cytokines increases insulin resistance [92]. Since *Nucb2* expression levels are abundant in immune cells and are increased in immune cells during high fat feeding, it is thus important to determine if *Nucb2* plays a regulatory role in immune cell activation or proliferation, and if this high fat induced expression of *Nucb2* leads to regulation of cytokine production.

The current findings, in conjunction with previous literature, leads to the hypothesis that increases in *Nucb2* expression in macrophages and T cells under conditions of high fat feeding serve as a regulatory mechanism to dampen inflammation. Thus, under conditions of high fat

feeding and in the absence of *Nucb2*, the knockout mice should be unable to adapt to obesity-induced immune cell inflammatory cytokine production, ultimately leading to insulin resistance. While our results show that *Nucb2* is clearly important in obesity induced metabolic dysregulation of insulin signaling and glucose metabolism, we have yet to determine if *Nucb2* is affecting the insulin receptor directly or other potential glucose transport proteins involved in glucose uptake into tissues.

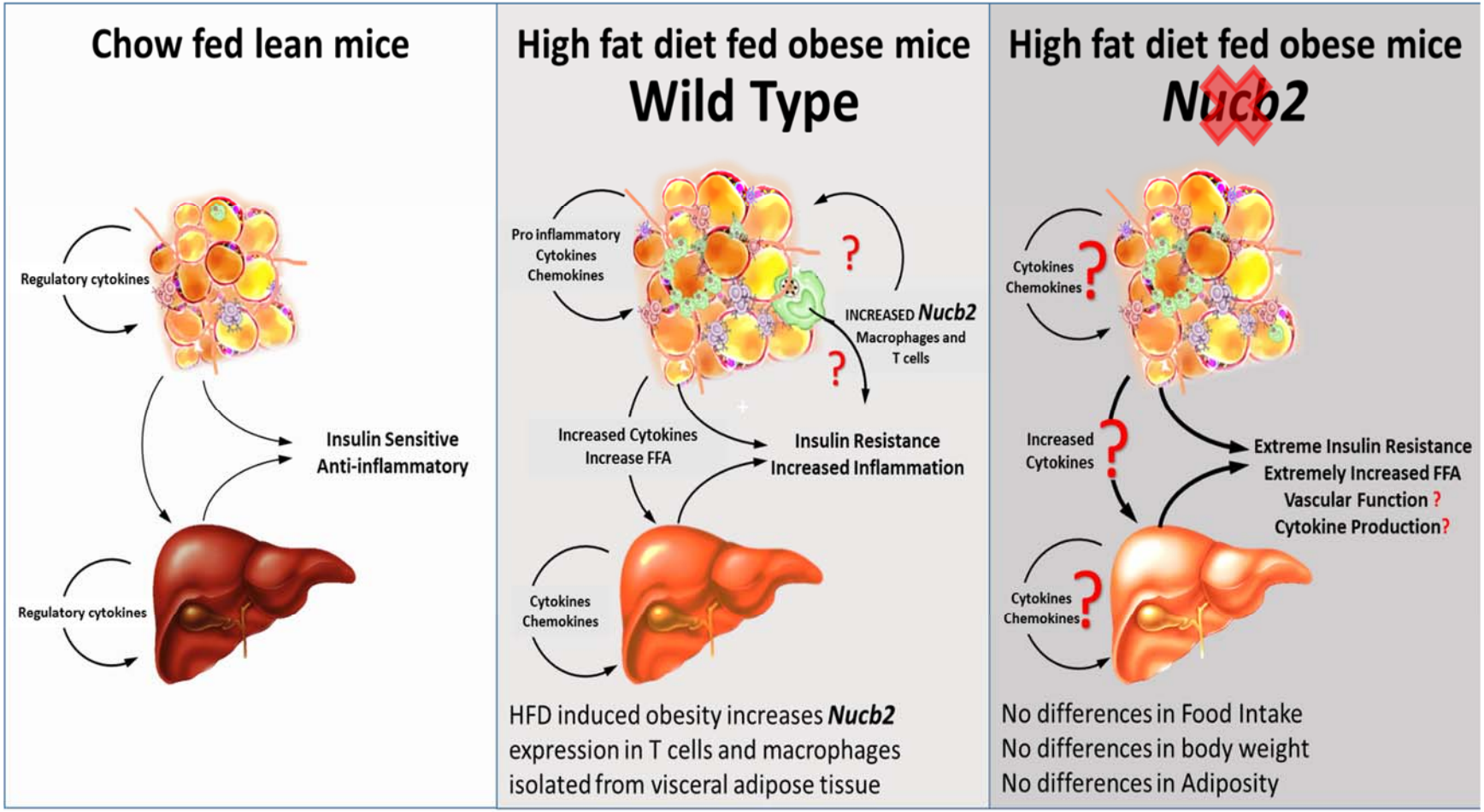


Figure 2.18: Major findings and conclusions from Chapter 2

Ablation of *Nucb2* renders mice to become extremely insulin resistant during high fat diet induced obesity. From our clamp data, it is evident that the *Nucb2-/-* mice have insulin resistance in adipocytes and hepatocytes. Mice lacking *Nucb2* are unable to shut down hepatic glucose production or lipolysis under insulin induced clamped conditions.

Chapter 3: Effects of Nucleobindin-2 on Myeloid Cells and Lymphocytes and Sterile Obesity-induced Inflammation

3.1 Introduction

With excess energy stores as lipids in adipocytes, obesity-related low-grade systemic inflammation contributes to the pathogenesis of at least seven out of ten of the leading causes of mortality. These include type 2 diabetes, heart disease, some forms of cancer, chronic lower respiratory disease, stroke, Alzheimer's disease, and kidney disease [7-13]. In a classical immunologic response, inflammation is characterized as an acute response to tissue damage or pathogen associated molecular patterns (PAMPs). These immunologic responses are necessary for host defense against infections and tissue remodeling in response to tissue injury. However, obesity-related sterile inflammation is not associated with overt infections and is a result of the activation of an immune response to self (increases in FFA's seen in obese) or typically innocuous stimuli (extracellular ATP or cholesterol crystals) in absence of infections. In obesity and aging, this sterile inflammation is hypothesized to be a fundamental cause for disruption of normal metabolic function which over time leads to degenerative diseases.

Over the years, more appreciation has been given to the link between inflammation and the pathogenesis of type 2 diabetes. Together with the discovery of the importance of adipose tissue leukocytosis, new findings are helping to explain how obesity increases the risk for developing insulin resistance, some facets of the metabolic syndrome and eventually diabetes. In 2003, Ferrante et al. showed that obesity is positively correlated with macrophage influx in adipose tissue which alters metabolic and endocrine function [158]. They showed that adipose tissue macrophages were responsible for almost all adipose tissue TNA-α expression and significant amounts of iNOS and IL-6 expression.

Hotamisligil et al. (1993) initially demonstrated that the proinflammatory cytokine TNF-α is able to directly induce insulin resistance [6]. The concept that adipose tissue was a site for the production of cytokines and hormones extended beyond TNF-α to include IL-1β, IL-6, leptin, monocyte chemoattractant protein-1, PAI-1, serum amyloid A and others [17, 159-161]. While some of these factors are true adipokines, many of these molecules are expressed at high levels in activated macrophages or other immune cell subtypes. Regardless, they undoubtedly participate in the induction and maintenance of the subacute inflammatory state associated with obesity and type 2 diabetes.

Ever evolving is the concept that insulin resistance and diabetes have immunological components. Moreover, improving knowledge of how inflammation modulates metabolism may provide new opportunities for using anti-inflammatory strategies as a therapy for the different

impairments occurring with the cardiometabolic syndrome progressively occurring with excess adiposity.

The studies described in Chapter 2 showed *Nucb2* expression was increased in adipose tissue macrophages and T cells from mice fed a HFD. We were thus interested in further exploring what role *Nucb2* might be playing in these immune cell subsets. To further delve into the mechanisms underlying the insulin resistance phenotype seen our my *Nucb2-/-* mouse model, we set out to test if *Nucb2* is important in immune cell activation and/or chronic, low grade systemic inflammation that commonly accompanies gains in adipose tissue in obesity. To do this, we studied the changes in immune cell activation via *in-vitro* cultures of femur bone marrow derived macrophages and *in vivo* gene expression models.

3.2 Materials and Methods

3.2.1 Cell isolation, flow cytometry and Fluorescence-Activated Cell Sorting (FACS)

Cells were isolated from adipose tissues using FACS technique. Antibodies used were CD3, F4/80, and B220 (eBioscience Affymetrix). Cells were acquired on a BD FACSCalibur, and data were analyzed in FlowJo (Treestar Inc., Ashland, OR, USA). To identify naïve and effector/memory T cells, splenocytes and Stromal Vascular Fraction (SVF) were incubated with fluorescently labeled PerCP-conjugated anti-CD4, APC-conjugated anti-CD8, PE-conjugated anti-CD62L, and FITC-conjugated anti-CD44 antibodies. SVF was also stained with anti-F4/80, CD11b, CD206 (eBiosciences, San Diego, CA, USA and Biolegend, San Diego, CA, USA) to assess macrophage and granulocyte populations. Data were acquired on a BD LSR II and analyzed using FlowJo.

3.2.2 Leukocyte quantification in adipose tissue

Leukocytes were quantified in inguinal and epididymal fat pads using flow cytometry analysis as previously described by our laboratory (Quantification of Adipose Tissue Leukocytosis in Obesity) [147]. Cells were labeled using antibodies described below, and cells were run on a FACSCalibur (BD Biosciences, San Jose, CA, USA). Macrophage subsets were identified using F4/80, CD206 and CD11c antibodies (eBiosciences, San Diego, CA, USA and Biolegend, San Diego, CA, USA). T cell subpopulations were identified by CD3, CD4 and CD8 antibodies, while B cell populations were identified by B220 antibodies for B220, IgM and CD19 (eBiosciences). FACS data were analyzed by post-collection compensation using FlowJo software (Treestar Inc., Ashland, OR).

3.2.3 Cell cultures

[All steps were performed using sterile technique in a laminar flow hood] Mouse femurs were collected in RPMI (22400105; Life Technologies) + 10% FBS (R10; Omega Scientific) + 1% Antibiotic-antimycotic (15240062; Gibco Thermofisher). Both ends of the femur were then cut

and the femur was flushed with R10. The bone marrow was centrifuged at 450 g for 5 min, the supernatant was decanted and red blood cells were lysed using ACK lysis buffer (118-156-101; Quality Biological). After neutralization with R10, bone marrow cells were centrifuged, suspended in 10 ml of R10 and placed into a six-well plate. Non-adherent cells were collected the following morning. The non-adherent cells were resuspended at 4 × 106 cells per ml in medium consisting of 10 ml supernatant of non-adherent cells, 7.2 ml L929 conditioned medium, 6.8 ml R10 and MCSF (10 ng/ml; 416-ML; R&D Systems). After 4 days, an additional 2 ml of fresh medium was added. Non-adherent cells were collected on day 7, separated by density gradient separation using Fico/Lite (I40650; Atlanta Biologicals) and mononuclear cells were collected. Cells were rinsed twice with Dulbecco's PBS + 2% FBS, and resuspended at 1×10^6 cells per ml. Cells were treated with ultrapure LPS (L6529-1mg, Sigma-Aldrich, St Louis, MO, USA) alone for 4 hrs or in combination with 5 mM ATP (1A7699-1G; Sigma-Aldrich, St Louis, MO, USA) for 1 hr, 4 ng/uL IFN-γ, 1ng/ml IL-4, 200ug/ml silica for 5 hrs. During the course of this work, some of the macrophages were pre-treated for 12 hrs with 10 mM, 20 mM or 40 mM Bay-11-7085 compound (B5681; Sigma-Aldrich, St Louis, MO, USA), which irreversibly inhibits the TNFα-inducible phosphorylation of IκB-α (IC_{50} = 10 μM) without affecting the constitutive IκB-α phosphorylation at indicated concentrations and time. The cell supernatants and cell lysates were collected after treatment and analyzed for different proinflammatory markers either by western blot or quantitative PCR.

3.2.4 Western Blotting

Bone marrow derived macrophage cell lysates were prepared using RIPA buffer and immediately snap frozen in liquid nitrogen. Samples were vortexed every 10 min for 1 hr. Samples were centrifuged at 14,000 g for 15 min, the supernatant was collected and the protein concentration was determined using the DC Protein Assay (Bio-RAD). Antibodies to IL-1β (1:500, GTX74034; Genetex), NFκB p65 (1:1,000 8242s, Cell Signalling), phosphorylated-NFκB p65 (1:1000 3033S, Cell Signaling), and β-actin (1:1,000 4967L; Cell Signaling) were used at the dilutions specified by the manufacturer. The immune complexes were visualized by incubation with horseradish peroxidase-conjugated anti-rat (PI31470; Pierce) or anti-rabbit secondary antibody (PI31460, Pierce). Immuno-reactive bands were visualized by enhanced chemiluminescence (PI32209; Pierce). Densitometry analysis was performed using the ImageJ Gel Analysis tool, where gel background was also removed individually for each band.

3.2.5 Quantitative PCR

RNA from tissue and cells were isolated using an RNeasy Plus mini and micro kit (Qiagen; 74106 and 74034) according to the manufacturer's instructions. DNA digestion was performed on the colums using RNase-Free DNase according to manufacturer's instructions (79254; Qiagen). Following RNA purification, these samples were then used for iScript cDNA synthesis using a reverse transcriptase PCR kit (BIO-RAD; Hercules, CA, USA). Quantitative PCR was performed with the LightCycler 480 II (Roche Applied Science; Indianapolis, IN, USA) and Power SYBR Green

detection reagent (Applied Biosystems by Thermo Fischer Scientific; Woolston Warrington, UK). Primer sequences for transcripts encoding proteins involved in lipid and glucose metabolism were designed with Primer Express Software. For *Nucb2*, we used the forward primer sequence 5'-AAAACCTTGGCCTGTCTGAA-3' and the reverse primer sequence 5'-CATCGATAGGAACAGCTTCCA-3'. For CCL4, we used the forward primer sequence 5'-GAAACAGCAGGAAGTGGGAG-3' and the reverse primer sequence 5'-CATGAAGCTCTGCGTGTCTG-3'. For IL-1β, we used the forward primer sequence 5'-GGTCAAAGGTTTGGAAGCAG-3' and the reverse primer sequence 5'-TGTGAAATGCCACCTTTTGA-3'. For IL-6, we used the forward primer sequence 5'-ACCAGAGGAAATTTTCAATAGGC-3' and the reverse primer sequence 5'-TGATGCACTTGCAGAAAACA-3'. For TNF-α, we used the forward primer sequence 5'-AGGGTCTGGGCCATAGAACT-3' and the reverse primer sequence 5'-CCACCACGCTCTTCTGTCTAC-3'. For MIP-1α, we used the forward primer sequence 5'-ACCATGACACTCTGCAACCA-3' and the reverse primer sequence 5'-GTGGAATCTTCCGGCTGTAG-3'.

In all qRT-PCR experiments, 25 ng cDNA was used. Fold induction of gene expression with *Nucb2* was analyzed with the ΔΔCt method (also known as the comparative Ct method) as determined by the following equation: ΔΔCt = ΔCt treatment (WT NFκB inhibited - ΔCt control (WT control)). Here, the ΔCt is the Ct value for the sample treatment normalized to the endogenous housekeeping *GAPDH* transcript.

3.2.6 Whole Transcriptome Sequencing (mRNA-seq)

Optical density values of extracted RNA were measured using NanoDrop (Thermo Scientific) to confirm an A260:A280 ratio above 1.9. RNA integrity number (RIN) was measured using BioAnalyzer (Agilent) RNA 6000 Pico Kit to confirm RIN above 7.

Samples were then run by the Yale Center for Genome Analysis using Clontech ultra low-input Library Prep Kit V2 (#634899, Clontech Laboratories, Inc. Mountain View, CA, USA). Samples were sequenced using single-end 1x75 on the Illumina HiSeq2500 (Illumina Biotechnology, San Diego, CA, USA). Read counts were normalized using DESeq2 (R-package) in collaboration with Life & Medical Sciences Institute at the University of Bonn. To avoid spurious fold changes during differential expression analysis, all normalized counts were set to a value of at least 1.

3.3 Results

3.3.1 *Nucb2* is not required for immune cell chemotaxis or adipose tissue leukocytosis

Previous studies have shown that mice fed a HFD recruit innate and adaptive immune cells in their epididymal white adipose tissue as they gain weight [22, 103, 104, 158]. Using fluorescence-activated cell sorting (FACS), we were able to quantitatively assess the kinetics of the immune cell subsets that infiltrate the different adipose tissue depots in mice fed different diets.

In mice fed a HFD, there was no difference in the immune cell subset frequencies within the adipose tissues. In inguinal adipose tissue, the myeloid lineage cells represented 11.4 ± 1.5% and 12.3 ± 0.4% in wild type and Nucb2 -/- mice, respectively (p=0.5), whereas macrophage populations were similarly unchanged between wild type and *Nucb2-/-* mice (16.7 ± 1.5% and 14.6 ± 0.9%, respectively, p=0.2). There were also no differences in myeloid cells (WT and KO: 11.8 ± 1.6% and 11.5 ± 0.7%, respectively, p=0.9) or macrophages (22.8 ± 4.4% and 23.1 ± 1.6%, respectively, p=0.1) frequencies in visceral mesenteric adipose tissue. Similarly, no differences were detected in visceral perirenal adipose tissue myeloid (WT and KO: 10.4 ± 0.6% and 10.7 ± 1.3%, respectively, p=0.8) and macrophages (WT and KO: 16.6 ± 2.7% and 19.8 ± 1.3%, respectively, p=0.3) (Figure 3.1).

In addition to no differences in myeloid lineage cells in different adipose tissue depots, there was also no difference in frequencies of lymphocyte populations. In inguinal adipose tissue, there were no differences in lymphocyte frequencies between wild type and *Nucb2-/-* mice (44.3 ± 3.2% and 48.2 ± 3.7%, respectively, p=0.5), in CD4+ helper T cells (12.1 ± 1.9% and 10.6 ± 1.3%, respectively, p=0.5) and in CD8+ cytotoxic T cells (12.3 ± 1.5% and 13.7 ± 1.1%, respectively, p=0.5). T cells were further divided into different subsets of CD4+ and CD8+ cells. To further characterize the subset of T cells in the inguinal adipose tissue, we studied potential differences in the prevalence of naïve and effector/memory cells. To identify these cells, we sorted the cells for cell surface glycoproteins such as CD44 and cell adhesion molecule CD62L (L-selectin). Even with these extra characterizations, we saw no differences between wild type mice and *Nucb2-/-* mice. CD4 naïve cell frequencies were similar between wild type and *Nucb2-/-* mice (2.3 ± 0.4% and 3.3 ± 1.0%, respectively, p=0.5), as were CD4 effector/memory cells (37.0 ± 2.8% and 36.3 ± 1.7%, respectively, p=0.8). CD8 naïve cell frequencies were 1.9 ± 0.2% and 2.4 ± 0.6% for wild type and *Nucb2-/-* respectively (p=0.5), and CD8 effector/memory cell frequencies were 46.3 ± 4.11% and 42.8 ± 2.2% in wild type and *Nucb2-/-* respectively, p=0.4 (Figure 3.2). These data show that there are no differences in adipose tissue leukocytosis between wild type and *Nucb2-/-* mice, and that *Nucb2* is not required for leukocyte chemotaxis.

In visceral mesenteric adipose tissue, frequencies of lymphocytes (50.8 ± 9.27% and 61.3 ± 3.23%, respectively, p=0.2), CD4+ helper T cells (13.4 ± 2.0% and 15.2 ± 1.5%, respectively,

p=0.5) and CD8+ cytotoxic T cells (8.7 ± 2.8% and 11.2 ± 1.5%, respectively, p=0.4) were all similar between wild type and *Nucb2-/-* mice. Similarly to inguinal adipose tissue, there were no differences between naïve and effector/memory cells in the mesenteric depot. CD4 naïve cell frequencies were 3.8% ± 1.4% and 3.6 ± 0.6% for wild type and *Nucb2-/-* animals, respectively (p=0.9). Interestingly, CD4 effector/memory cell frequencies were 47.1 ± 5.5% and 57.4 ± 1.9% with increases seen in *Nucb2-/-* mice (p=0.05). CD8 naïve cell frequencies were 3.2 ± 0.9% and 2.9 ± 0.5% for wild type and *Nucb2-/-* respectively (p=0.8) and CD8 effector/memory cell frequencies were 45.0 ± 4.8% and 48.8 ± 1.8%, wild type and *Nucb2-/-* respectively, p=0.4 (Figure 3.3).

In visceral perirenal adipose tissue, there were also no differences in lymphocyte frequencies between wild type and *Nucb2-/-* mice (25.8 ± 6.5% and 19.2 ± 4.1%, respectively, p=0.4), CD4+ helper T cells (10.4 ± 0.9% and 7.7 ± 1.5%, respectively, p=0.2) and CD8+ cytotoxic T cells (13.1 ± 2.7% and 16.9 ± 2.1%, respectively, p=0.3). As in other adipose depots, there were no differences between naïve and effector/memory cells in the perirenal depot. CD4 naïve cell frequencies were 5.9% ± 1.9% and 3.8 ± 0.9% for wild type and *Nucb2-/-* mice, respectively (p=0.3), and CD4 effector/memory cell frequencies were 37.4 ± 2.7% and 41.0 ± 4.4%, for wild type and *Nucb2-/-* animals, respectively, p=0.6. CD8 naïve cell frequencies were 1.5 ± 0.7% and 1.2 ± 0.3% for wild type and *Nucb2-/-* mice, respectively (p=0.5), and CD8 effector/memory cell frequencies were 47.3 ± 4.7% and 48.8 ± 2.2%, wild type and *Nucb2-/-* animals, respectively, p=0.7(Figure 3.4).

Overall, there were no differences in immune cell leukocytosis in the different adipose tissues examined. There were also no differences in frequencies of both myeloid and lymphoid lineage cells in the adipose tissue depots harvested from mice fed a high fat diet. This is imperative to conclude that *Nucb2* does not have an effect on chemotaxis of immune cells to tissues. In conclusion, the migration and differentiation of immune cell subsets were not affected by ablation of the *Nucb2* gene.

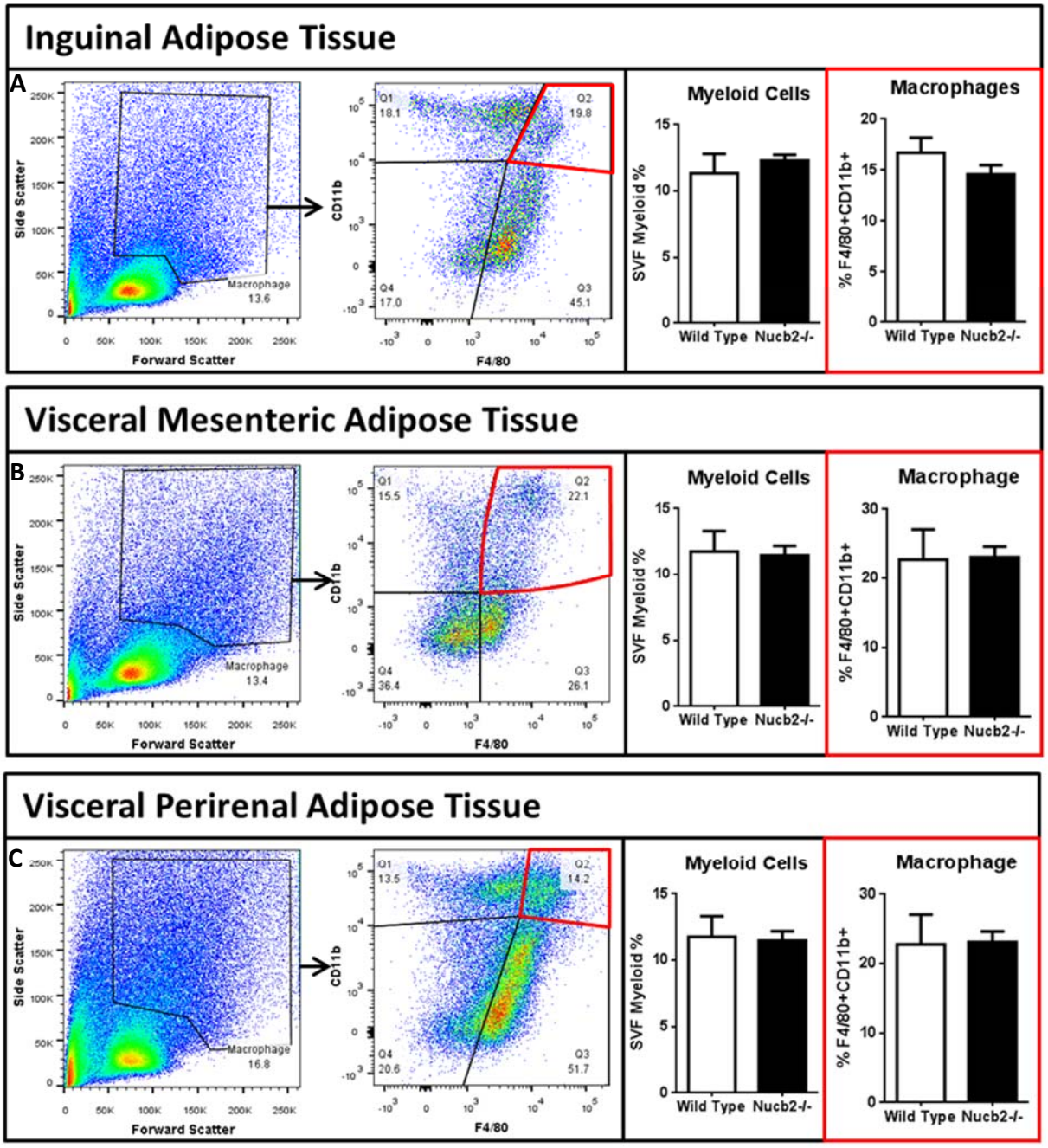


Figure 3.1: *Nucb2* ablation does not impact the number of macrophages in adipose tissue

Representative flow cytometry dot plots of SVF from subcutaneous inguinal adipose tissue, visceral mesenteric adipose tissue and visceral perirenal adipose tissue with corresponding quantitation. Macrophage populations in (A) Inguinal adipose tissue gating strategy and macrophage quantifications, (B) Visceral mesenteric adipose tissue gating strategy and macrophage quantifications and (C) Visceral perirenal adipose tissue gating strategy and macrophage quantifications. All data are represented as mean ± SEM (mice are 8 month old, male high fat diet fed wild type, n=5 and *Nucb2 -/-,* n=8)

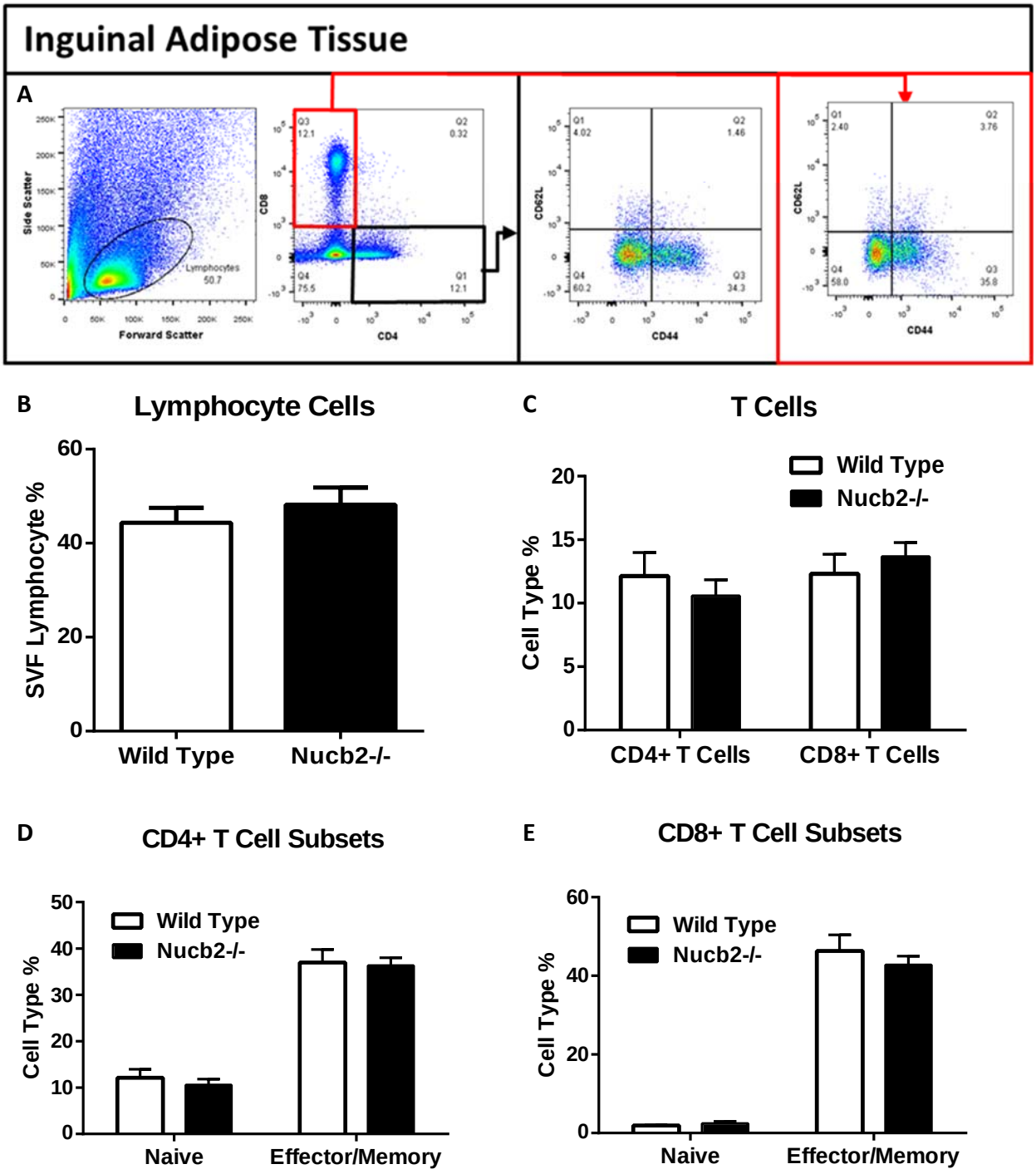


Figure 3.2: *Nucb2* ablation does not affect inguinal adipose tissue lymphocyte nor T cell subset frequency

Lymphoid populations in inguinal adipose tissue. (A) Flow gating strategy for the lymphoid T cell subset analysis (B) Lymphoid cell quantification (C) CD4 and CD8 positive T cell subset quantification (D) CD4+ naïve and effector/memory quantifications and (E) CD8+ naïve and effector/memory quantifications. All data are represented as mean ± SEM (mice are 8 month old, male high fat diet fed wild type, n=5 and *Nucb2 -/-,* n=8)

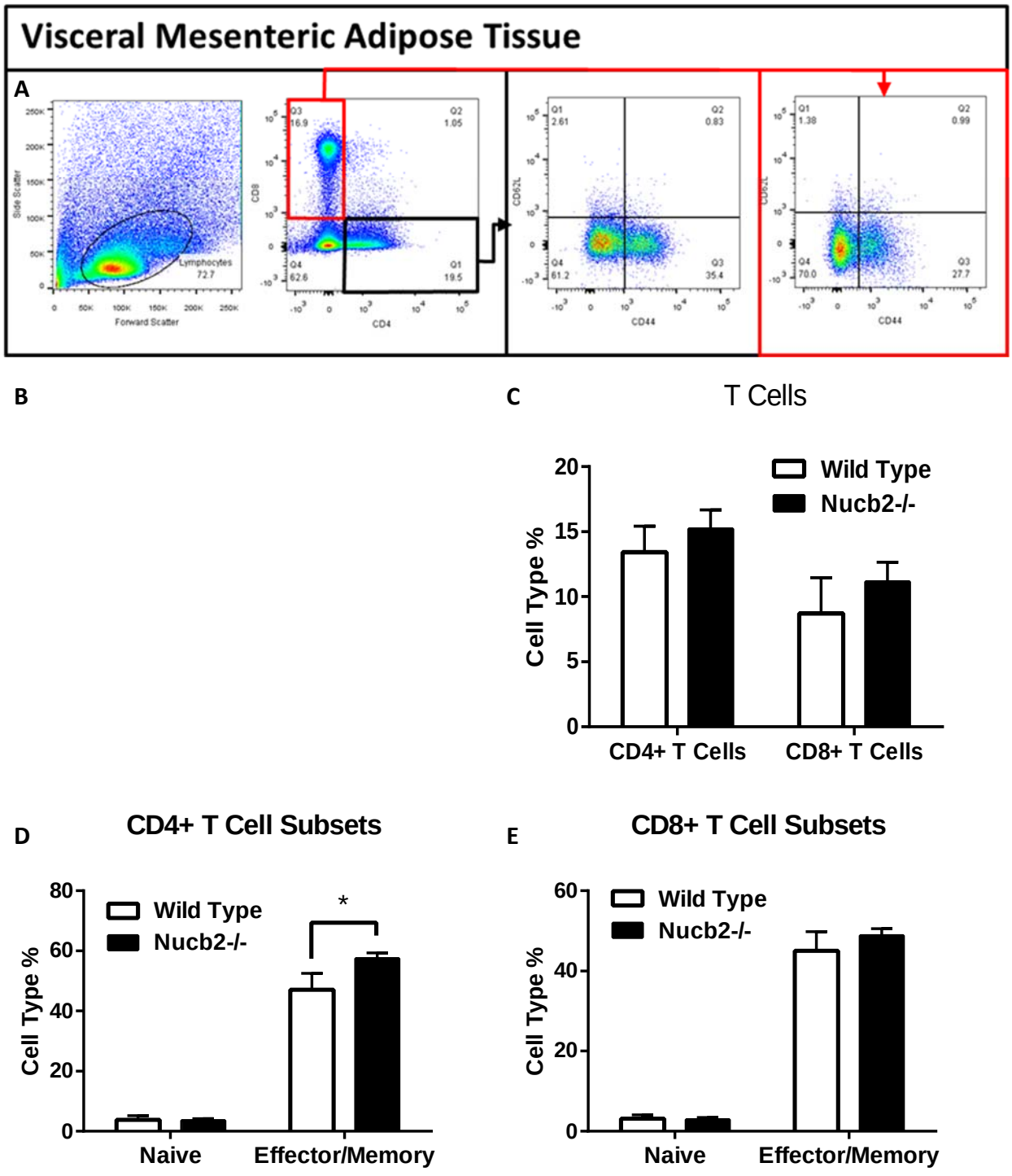


Figure 3.3: *Nucb2* ablation does not affect visceral mesenteric adipose tissue lymphocyte nor T cell subset frequency

Lymphoid populations in mesenteric adipose tissue. (A) Flow gating strategy for the lymphoid T cell subset analysis (B) Lymphoid cell quantification (C) CD4 and CD8 positive T cell subset quantification (D) CD4+ naïve and effector/memory quantifications and (E) CD8+ naïve and effector/memory quantifications. All data are represented as mean ± SEM (mice are 8 month old, male high fat diet fed wild type, n=5 and *Nucb2 -/-,* n=8)

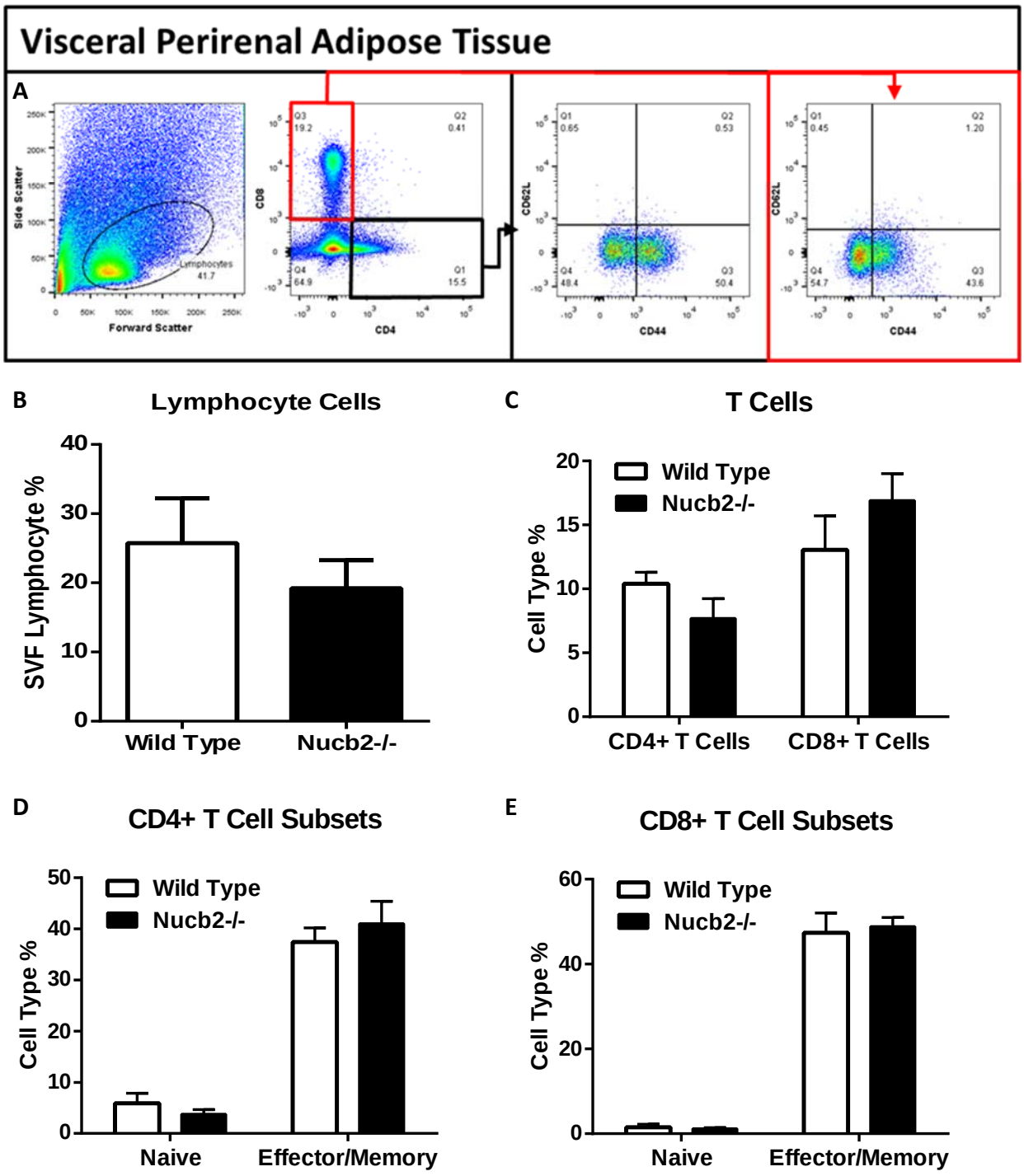


Figure 3.4: *Nucb2* ablation does not affect visceral perirenal adipose tissue lymphocyte nor T cell subset frequency

Lymphoid populations in visceral perirenal adipose tissue. (A) Flow gating strategy for the lymphoid T cell subset analysis (B) Lymphoid cell quantification (C) CD4 and CD8 positive T cell subset quantification (D) CD4+ naïve and effector/memory quantifications and (E) CD8+ naïve and effector/memory quantifications. All data are represented as mean ± SEM (mice are 8 month old, male high fat diet fed wild type, n=5 and *Nucb2 -/-,* n=8)

3.3.2 *Nucb2* is important for regulation of proinflammatory cytokine production in classically activated M1-like macrophages

Since *Nucb2* is highly expressed in leukocytes, we next determined if there were differences in macrophage sub-population activation state and/or polarization. Classically activated (M1) macrophages are predominantly proinflammatory, are dependent on TLRs, and have the role of effector cells in Th1 cellular immune responses. The alternatively activated (M2) macrophages are involved in immunosuppression and tissue repair and are considered more anti-inflammatory. The outer membrane of Gram-negative bacteria, endotoxin lipopolysaccharide (LPS) and the Th1 cytokine IFN-γ polarize macrophages towards the M1 phenotype, inducing the macrophages to produce large amounts of proinflammatory cytokines. Using an *in vitro* bone marrow derived macrophage (BMDM) model, we tested whether *Nucb2* is required for normal macrophage function in chosen macrophage subsets, and determined if *Nucb2* partially regulates inflammatory cytokine production.

There were no differences in proinflammatory cytokine production between wild type and *Nucb2 -/-* mice in basal, unpolarized M0 macrophages. As expected, however, there was a significant increase in proinflammatory cytokine production in M0 vs. M1 polarized macrophages in both wild type and *Nucb2-/-* mice. Intriguingly, *Nucb2-/-* "M1" polarized macrophages had significant increases in relative fold change expression of TNF-α (WT and *Nucb2-/-*: 5.8 ± 0.3 and 9.7 ± 0.3, respectively, p=0.0001), IL-1β (WT and *Nucb2-/-*: 9.1 ± 1.5 and 23.9 ± 1.7, respectively, p=0.0006), MIP-1α (WT and *Nucb2-/-*: 1.2 ± 0.1 and 2.4 ± 0.3, respectively, p=0.01) and CCL4 (WT and *Nucb2-/-*: 1.4 ± 0.0 and 2.4 ± 0.3, respectively, p=0.007) (Figure 3.5). To validate our skewing of macrophages, we measured classical markers of M1 macrophages (iNOS) and M2 macrophages (Arginase-1). Relative expression of iNOS was significantly increased in M1 skewed macrophages, with even more elevated levels in *Nucb2-/-* macrophages when compared to WT (WT and *Nucb2-/-:* 5.8 ± 0.3 and 9.7 ± 0.3, respectively, p=0.0001).

To confirm this increase in proinflammatory cytokine production, we conducted several experiments on "M1" polarized macrophages to evaluate proinflammatory cytokine production. The above outcomes were corroborated many times, showing *Nucb2-/-* "M1" polarized macrophages have significant increases in cytokine production rates compared to wild type controls. Fold change of the relative expression of TNF-α (WT and *Nucb2-/-*: 1.0 ± 0.0 and 1.9 ± 0.1, respectively, p=0.0003), IL-1β (WT and *Nucb2-/-*: 1.0 ± 0.1 and 1.7 ± 0.1, respectively, p=0.0003), MIP-1α (WT and *Nucb2-/-*: 1.0 ± 0.04 and 2.0 ± 0.19, respectively, p=0.002), CCL4 (WT and *Nucb2-/-*: 1.0 ± 0.0 and 1.8 ± 0.1, respectively) as well as iNOS (WT and *Nucb2-/-*: 1.0 ± 0.0 and 1.6 ± 0.1, respectively, p<0.0001) were all increased in *Nucb2* deficient macrophages vs. wild type macrophages. Nucb2 expression levels were verified as a control, and there was indeed no

gene expression of Nucb2 in the knock out BMDMs (WT and *Nucb2 -/-*: 1.0 ± 0.03 and 0.00002 ± 0.0001, respectively, p<0.0001) (Figure 3.6).

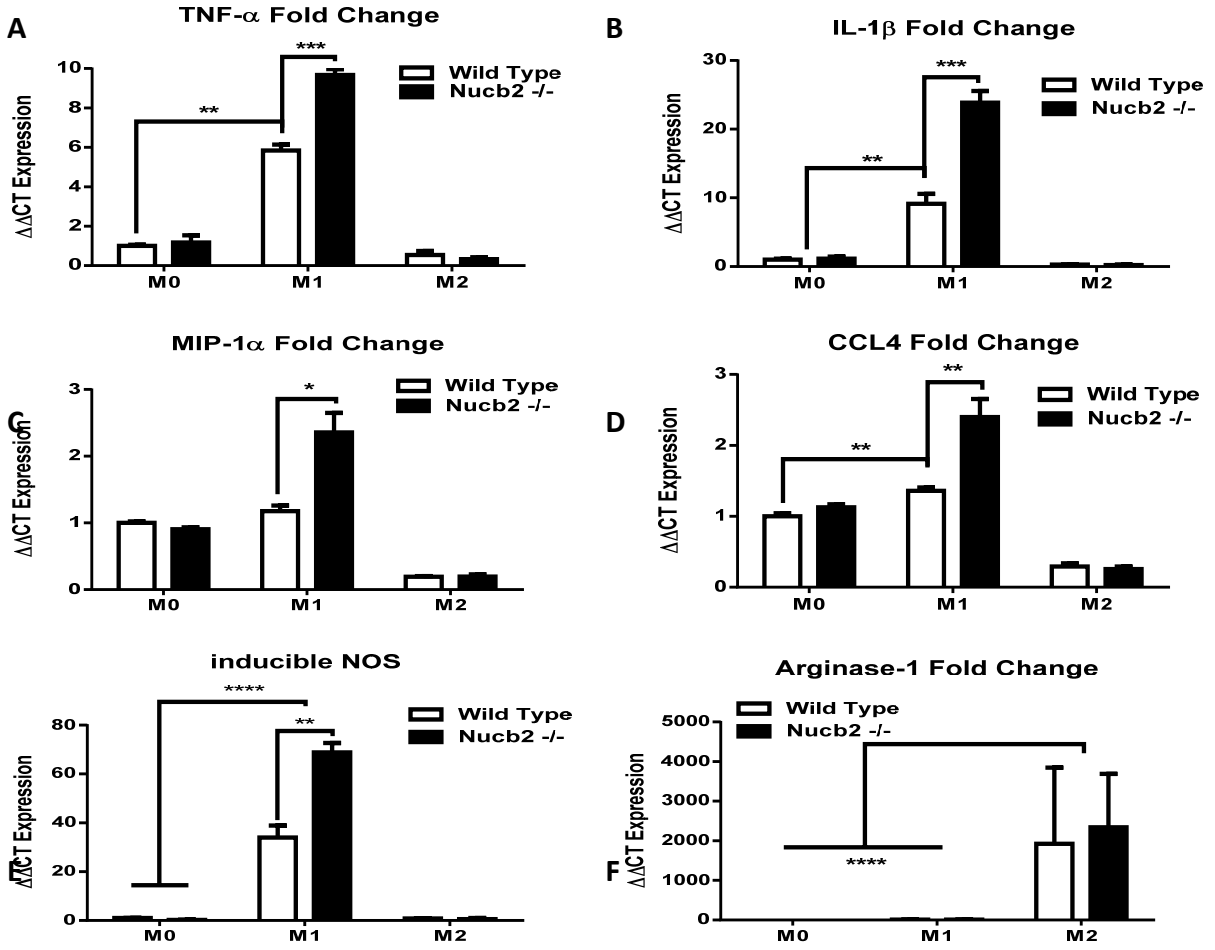


Figure 3.5: qRT-PCR to demonstrate *Nucb2* ablation impacts macrophage polarization and increases the expression of proinflammatory cytokines in M1-like BMDMs

Primary bone marrow derived macrophages (BMDMs) from wild type mice and *Nucb2 -/-* mice were treated with media alone for unpolarized "M0" macrophages, 1 ug/mL LPS plus 4 ng/mL interferon gamma (IFNγ) to drive the proinflammatory classically activated "M1" macrophages, or 1ng/ml IL-4 to drive anti-inflammatory alternatively activated "M2" macrophages . (A) TNF-α relative gene expression levels (B) IL-1β relative gene expression levels (C) MIP-1α relative gene expression levels and (D) CCL4 relative gene expression levels (E) inducible Nitric Oxide Synthase (iNOS) relative gene expression levels and (F) Arginase-1 relative gene expression levels. All data are represented as mean ± SEM (n=3/4 per group). *p< 0.05

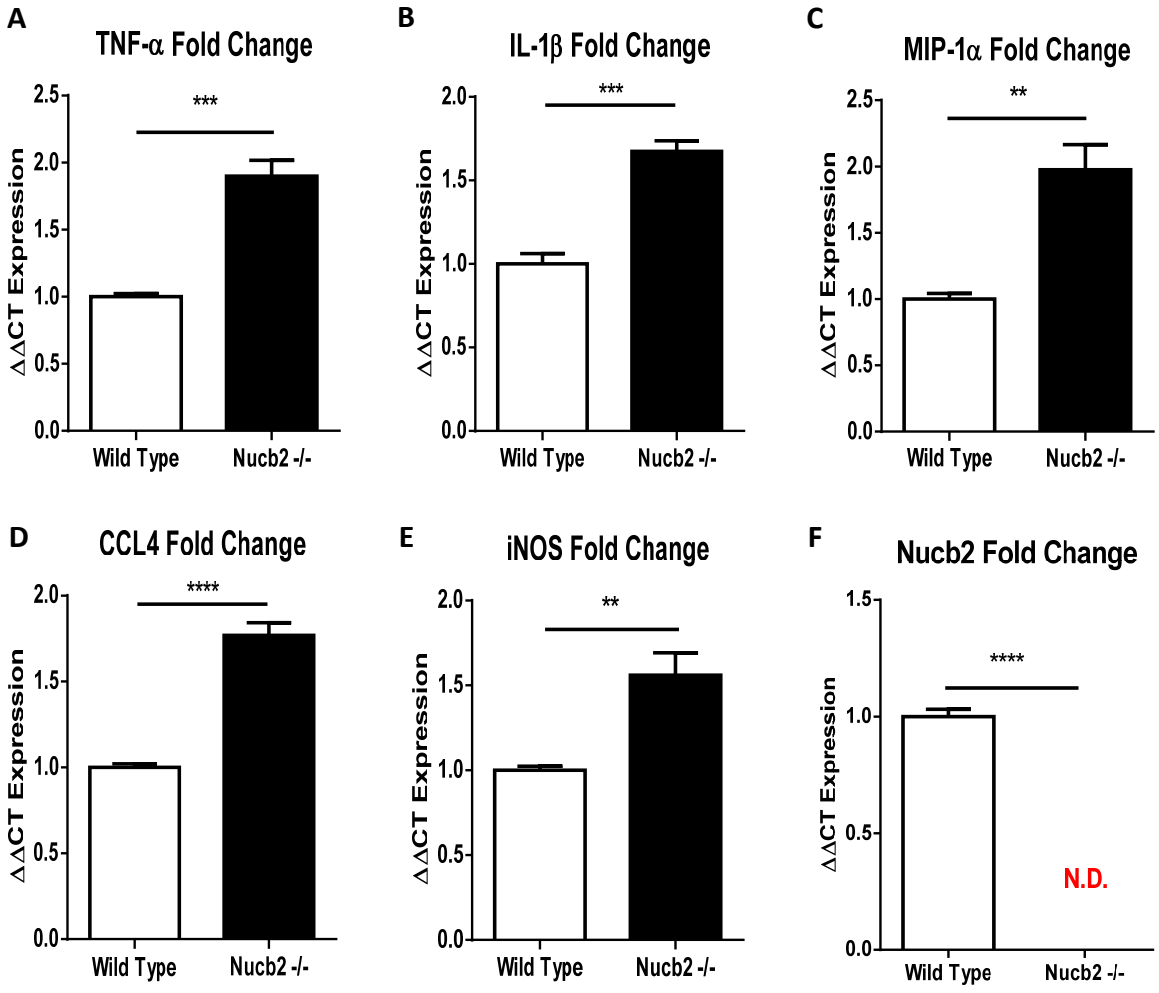


Figure 3.6: qRT-PCR to demonstrate *Nucb2* ablation increases the expression of proinflammatory cytokines in M1-like macrophages

Primary bone marrow derived macrophages (BMDM) from wild type mice and Nucb2 -/- mice were treated with media and 1 ug/mL LPS plus 4 ng/mL interferon gamma (IFNγ) to drive the proinflammatory "M1" phenotype. (A) TNF-α relative gene expression levels (B) IL-1β relative gene expression levels (C) MIP-1α relative gene expression levels (D) CCL4 relative gene expression levels (E) iNOS and (F) *Nucb2.* All data are represented as mean ± SEM (n=4 per group). *p< 0.05 relative to wild type

Taken together, our data indicate that *Nucb2* is required for regulating inflammatory cytokine production. Absence of *Nucb2* renders BMDM cells constitutively more skewed in a M1-like classically activated macrophage state with significantly increased levels of proinflammatory cytokines.

3.3.3 *Nucb2* regulates NFκB and IL-1β secretion in activated macrophages

IL-1β is sequestered as pro-IL-1β and requires post translational cleavage to become active. Pro-IL-1β can be cleaved and activated by the NLRP3 inflammasomes. NLRP3 is an important innate immune sensor that requires 2 different signals for activation and cleavage of pro- IL-1β and pro-IL-18. NFκB is a protein complex that controls transcription of inflammasomes NLRP3, as well as proinflammatory cytokine production and cell survival. In unstimulated cells, NFκB dimers are sequestered in the cytoplasm by a family of inhibitors. However, when activated by a cascade of enzymatic processes, active NFκB is translocated to the nucleus. LPS is a PAMP that activates TLR4 which in turn activates NFκB and further the transcription of inflammasome. Therefore, it is not surprising that NFκB is found to be chronically active in many inflammatory diseases is signal 1 for inflammasome activation. Second signal is in response to structurally diverse damage- DAMPs such as toxins, ATP, excess glucose, ceramides, amyloids, urate and cholesterol crystals and is necessary for the activation and NLRP3 complex formation.

To determine the mechanistic pathway by which *Nucb2* is working in the inflammatory cascade, we studied IL-1β protein production in supernatant, as well as NFκB in cell lysates. Ablation of *Nucb2* significantly increased protein levels of both IL-1β and NFκB, as assessed by western blotting. These results suggest that *Nucb2* has suppressive activity on NFκB and possibly on inflammasomes.

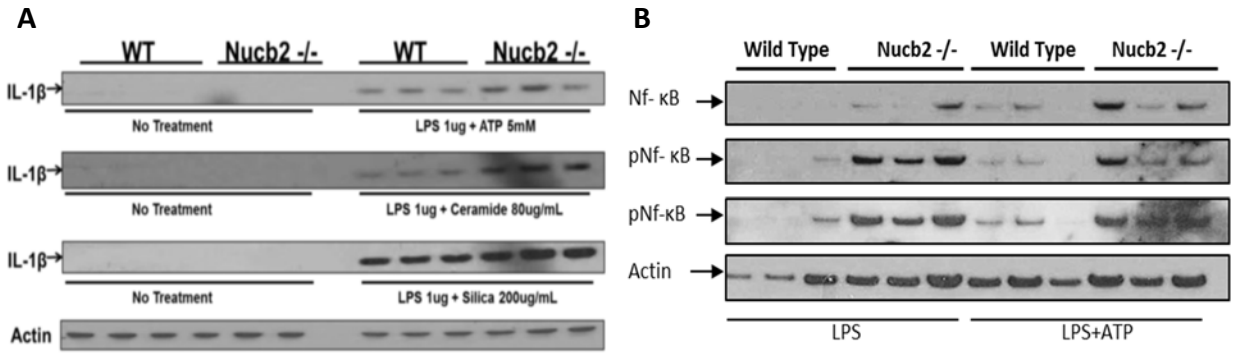


Figure 3.7: *Nucb2* ablation increases NFκB activation and IL-1β secretion in response to NLRP3 ligands

Representative Western blot analysis of (A) IL-1β (active p17) in the supernatant and (B) NFκB (p65) in the cell lysates of BMDMs primed with LPS for 4 h and stimulated with ATP for 1 h, Ceramide for 6 hrs or Silica for 5 hrs in wild type or *Nucb2-/-* derived cells.

Overall, the above data show that ablation of *Nucb2* constitutively increases protein levels of NFκB and active phosphorylated NFκB. Also, *Nucb2-/-* increases the production and secretion of activated proinflammatory IL-1β. These data indicate that *Nucb2-/-* BMDMs have increased

secretion of IL-1β in response to different stimuli. Furthermore, BMDMs lacking *Nucb2* exhibit considerably more signal 1 as seen by increased NFκB and phosphorylated NFκB (Figure 3.7). To further dissect mechanisms of *Nucb2,* It would be necessary to test if there is increased amounts of NLRP3 or other inflammasomes.

3.3.4 *Nucb2* regulates proinflammatory cytokine expression in macrophages via NFκB activation

M1 macrophage activation is dependent on TLRs and on activation of nuclear factor kappa B (NFκB) and c-Jun N-terminal kinase 1 (JNK1), leading to production of inflammatory cytokines, such as TNF-α and IL-1β. These cytokines in turn activate iNOS, resulting in increased production of reactive oxygen species such as nitric oxide (NO). To determine if the increased production of inflammatory cytokines seen in *Nucb2-/-* mice is dependent on the canonical NFκB pathway, we used an inhibitor of NFκB. The Bay-11-7085 NFκB inhibitor irreversibly impedes TNFα-inducible phosphorylation of IκB-α (IC50 = 10 μM) without affecting constitutive IκB-α phosphorylation. This in turn prevents the translocation of RelA/p50 to the nucleus and activation of the transcription of proinflammatory cytokines.

This experiment (Figure 3.8) indicated that the effects seen in *Nucb2 -/-* BMDMs are mediated by the NFκB pathway. When NFκB was blocked, the increases in proinflammatory cytokine production were all attenuated. Corroborating with our previous BMDM studies, *Nucb2-/-* mice without NFκB inhibitor have significantly augmented proinflammatory cytokine production compared to wild type controls. TNF-α relative expression levels were 2.27 ± 0.31 fold higher ($p=0.002$), IL-1β relative expression levels 3.6 ± 0.22 fold higher ($p<0.0001$), MIP-1α relative expression levels 2.69 ± 0.38 fold higher ($p=0.0009$) and CCL4 relative expression levels 2.47 ± 0.24 fold higher ($p=0.0002$) in *Nucb2 -/-* compared to wild type control mice. Remarkably, these increased levels of proinflammatory cytokines were almost completely attenuated when the NFκB pathway was inhibited by the Bay-11-7085 NFκB compound. When NFκB was inhibited, TNF-α relative expression levels were attenuated from 2.27 ± 0.31 to 1.21 ± 0.2 fold ($p=0.02$), IL-1β relative expression levels from 3.6 ± 0.22 to 1.6 ± 0.19 fold ($p=0.0001$), MIP-1α relative expression levels from 2.69 ± 0.38 to 1.27 ± 0.14 fold ($p=0.008$) and CCL4 relative expression levels from 2.47 ± 0.24 to 1.47 ± 0.1 fold ($p=0.006$) higher compared to wild type control mice (Figure 3.8). These data demonstrate that *Nucb2* regulates inflammation through the NFκB pathway.

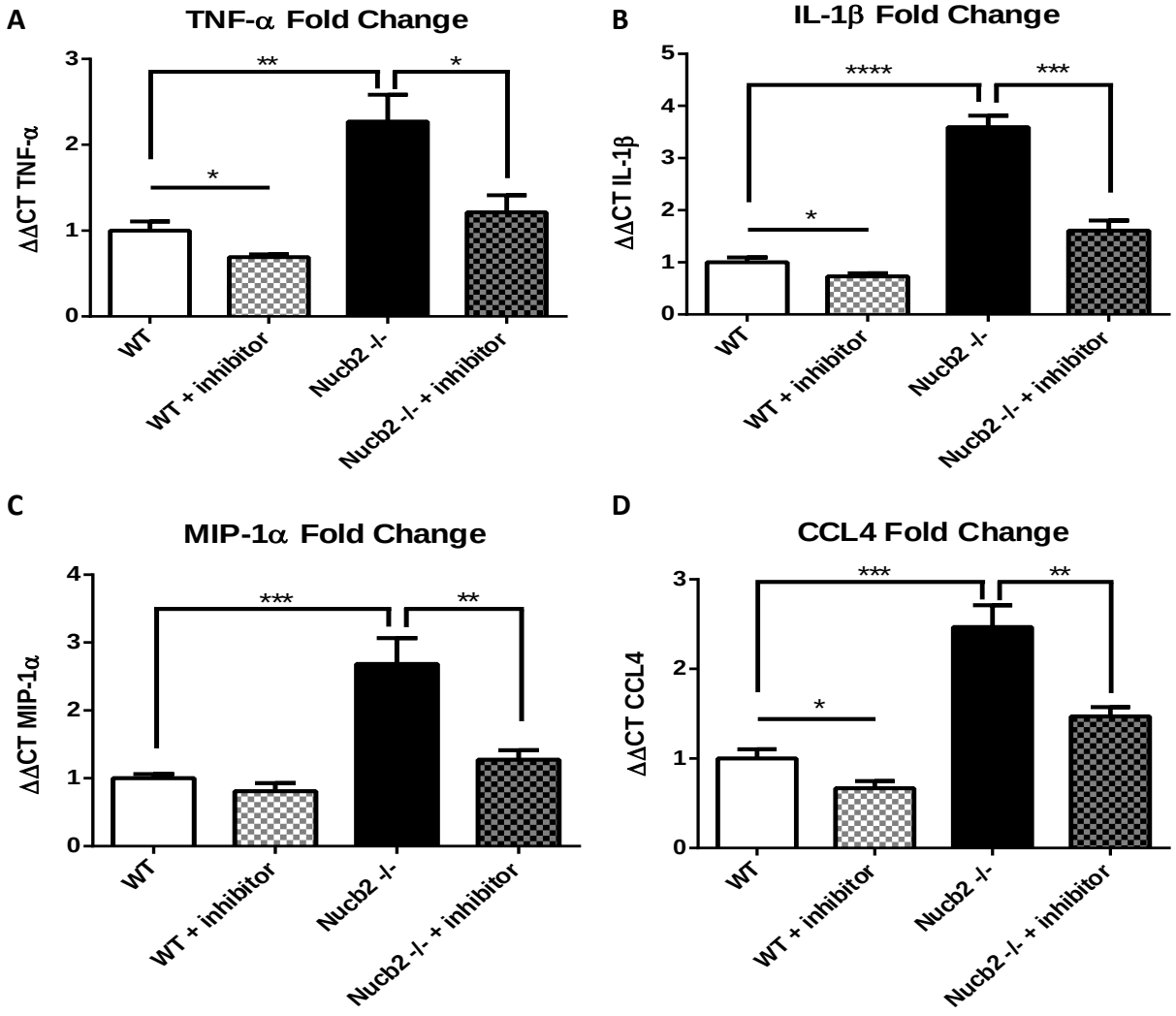


Figure 3.8: Regulation of pro-inflammatory cytokines by *Nucb2* is mediated by NFκB

Primary bone marrow derived macrophages (BMDMs) from wild type mice and Nucb2 -/- mice were treated with media and 1 ug/mL LPS plus 4 ng/mL interferon gamma (IFNγ) to drive the proinflammatory "M1" phenotype. Some macrophages were pretreated with Bay-11-7085 NFκB inhibitor. (A) TNF-α relative gene expression levels (B) IL-1β relative gene expression levels (C) MIP-1α relative gene expression levels and (D) CCL4 relative gene expression levels. All data are represented as mean ± SEM (n=6 per group). *p< 0.05 relative to wild type

3.3.5 Visceral adipose tissue macrophages from *Nucb2-/-* mice have increased proinflammatory cytokine production

As a model system to study the function of the highly diversified and complex cell type that is the macrophage, BMDMs are a good source for gene function studies and provide an abundant source of cells. However, it is important to note that differences in culturing technique can lead to phenotypic differences and functional disparities, including alterations in cell surface maturation markers and in the ability to secrete cytokines and phagocytose foreign materials

[162]. To eliminate this potential technical bias and examine whether the observed phenotypic differences and identified mechanism were physiologically relevant, we set out to determine if the above results were in fact replicable in an *in vivo* model. To do so, we used antibody against macrophage specific F4/80 cell surface glycoprotein and FACS sorted macrophages directly from the visceral adipose tissue of mice (*Nucb2 -/-* and WT control).

The increased proinflammatory state seen in BMDMs was also seen in isolated primary F4/80+, adipose tissue macrophages of 10 month old, HFD-fed mice that lack Nucb2. Macrophages sorted directly from *Nucb2-/-* adipose tissue had significant increases in all but one studied proinflammatory cytokine levels compared to wild type controls (Figure 3.9). TNF-α relative expression levels were 1.43 ± 0.04 fold higher (p=0.01), IL-1β 1.6 ± 0.16 fold (p=0.04), MIP-1α 1.83 ± 0.23 fold (p=0.009) and CCL4 1.44 ± 0.2 (p=0.08) fold higher in Nucb2 -/- mice than wild type controls.

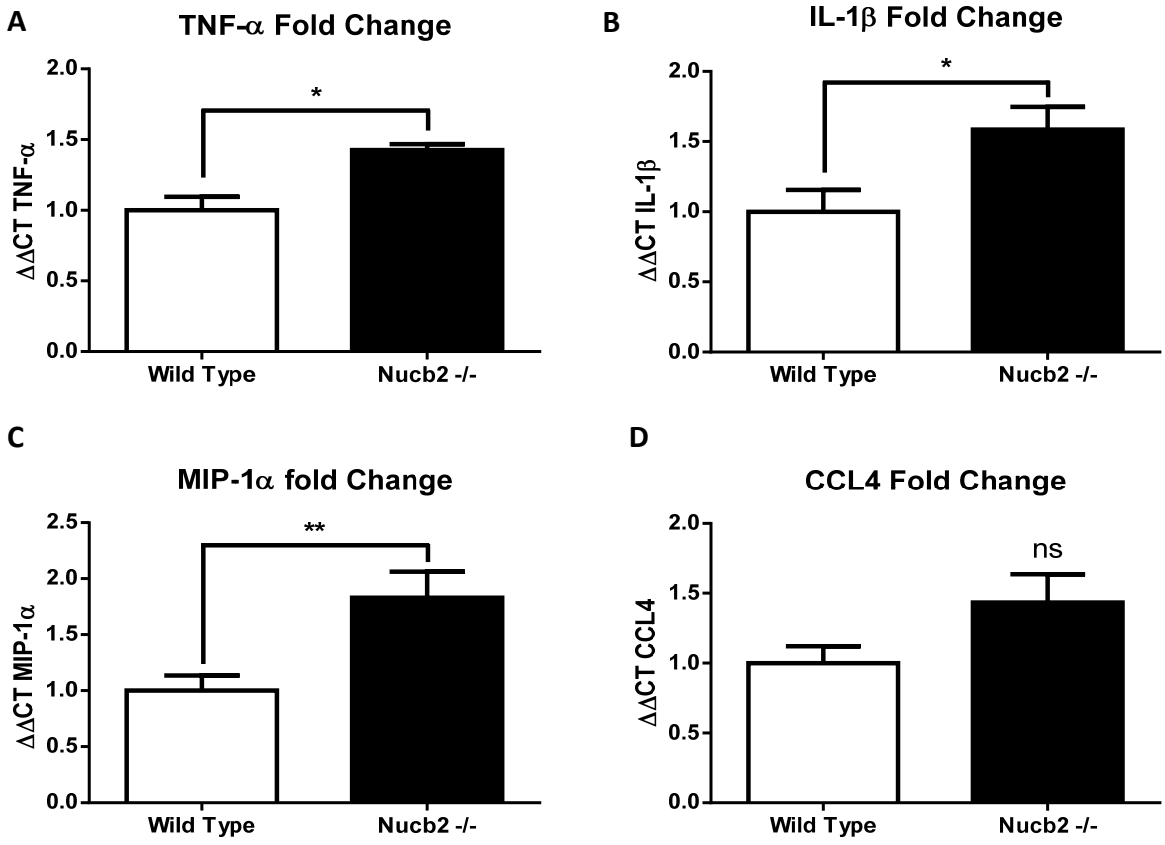


Figure 3.9: *Nucb2* ablation increases *in vivo* expression of inflammatory cytokines in obese adipose tissue macrophages

Relative cytokine expression levels from F4/80+ sorted cells from wild type and Nucb2 -/- mice. (A) TNF-α relative gene expression levels (B) IL-1β relative gene expression levels (C) MIP-1α relative gene expression levels and (D)

CCL4 relative gene expression levels. All data are represented as mean ± SEM (n=6 per group). *p< 0.05 relative to wild type

These data indicated that our *in vitro* BMDM studies are translated in an *in vivo* model. Increases in proinflammatory cytokines in the BMDMs are mimicked in macrophages isolated from visceral adipose tissue.

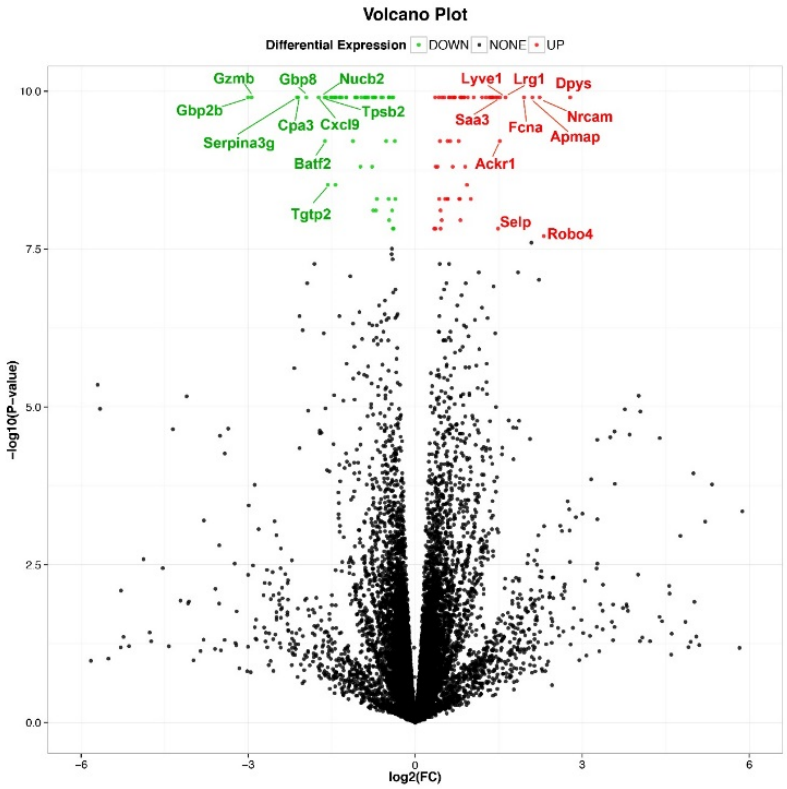
3.3.6 RNA sequencing of KO and WT adipose tissue macrophages shows impairments in Type I interferon signaling

To further investigate the changes induced by the knockout of *Nucb2* on the whole transcriptome of macrophages, we performed RNA-sequencing on F4/80+ isolated macrophages from visceral adipose tissue. Interestingly, a significant number of genes that were differentially regulated were interferon stimulated genes (ISG) (41 of the 122 genes). As well as a strong ISG difference, further analysis showed that several of the upregulated pathways in our model were those of more M1 like proinflammatory macrophages.

For example, our pathway analyses conveyed that macrophages from *Nucb2-/-* mice had a significant increase in tryptophan metabolism as well as glycolysis and gluconeogenesis pathways. In recent years, a clear association has been made between tryptophan catabolism and inflammatory reactions in a vast array of disease states [163]. Previous studies show that nitric oxide production and iNOS transcription rely heavily on tryptophan metabolism [164]. Other groups further went on to show that depletion of tryptophan via the rate limiting enzyme indoleamine 2,3-dioxygenase (IDO) is the mechanism by which activated macrophages inhibit T cell activation [165] and is necessary for immune tolerance.

In line with these macrophages being more inflammatory M1 cells is the fact that these *Nucb2-/-* macrophages have an increased glycolytic metabolism. It has been shown that inflammatory M1 macrophages display enhanced glycolytic metabolism and reduced mitochondrial activity and that conversely, anti-inflammatory M2 macrophages show a high mitochondrial oxidative phosphorylation state [166]. Furthermore, the stimulation of glycolysis is an activation signal for macrophages [167]. The fact that these pathways are upregulated in our macrophages from our Nucb2-/- mice when compared to wild type macrophages might elucidate a mechanism by which these macrophages are more inflammatory.

A



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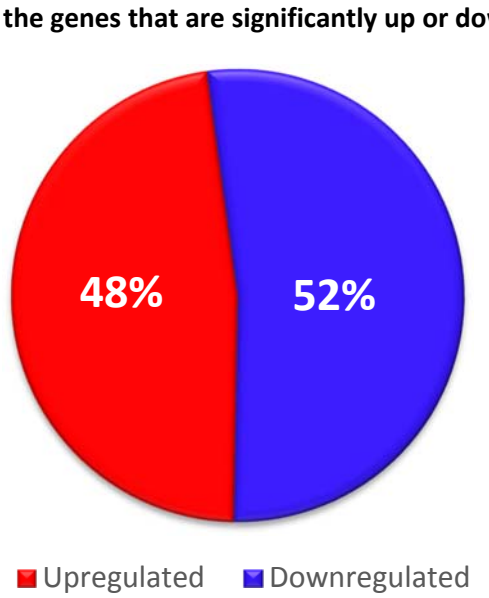


Figure 3.10: *Nucb2* ablation alters adipose tissue macrophage transcriptional signatures

RNA sequencing results of adipose tissue macrophages sorted from high fat diet fed *Nucb2-/-* mice and wild type mice. (A) Volcano plot with up (red) and down (green) regulated genes and (B) Pie chart expressing percentage of up (Red) and down (Blue) regulated genes. WT n=6, *Nucb2-/-* n=5

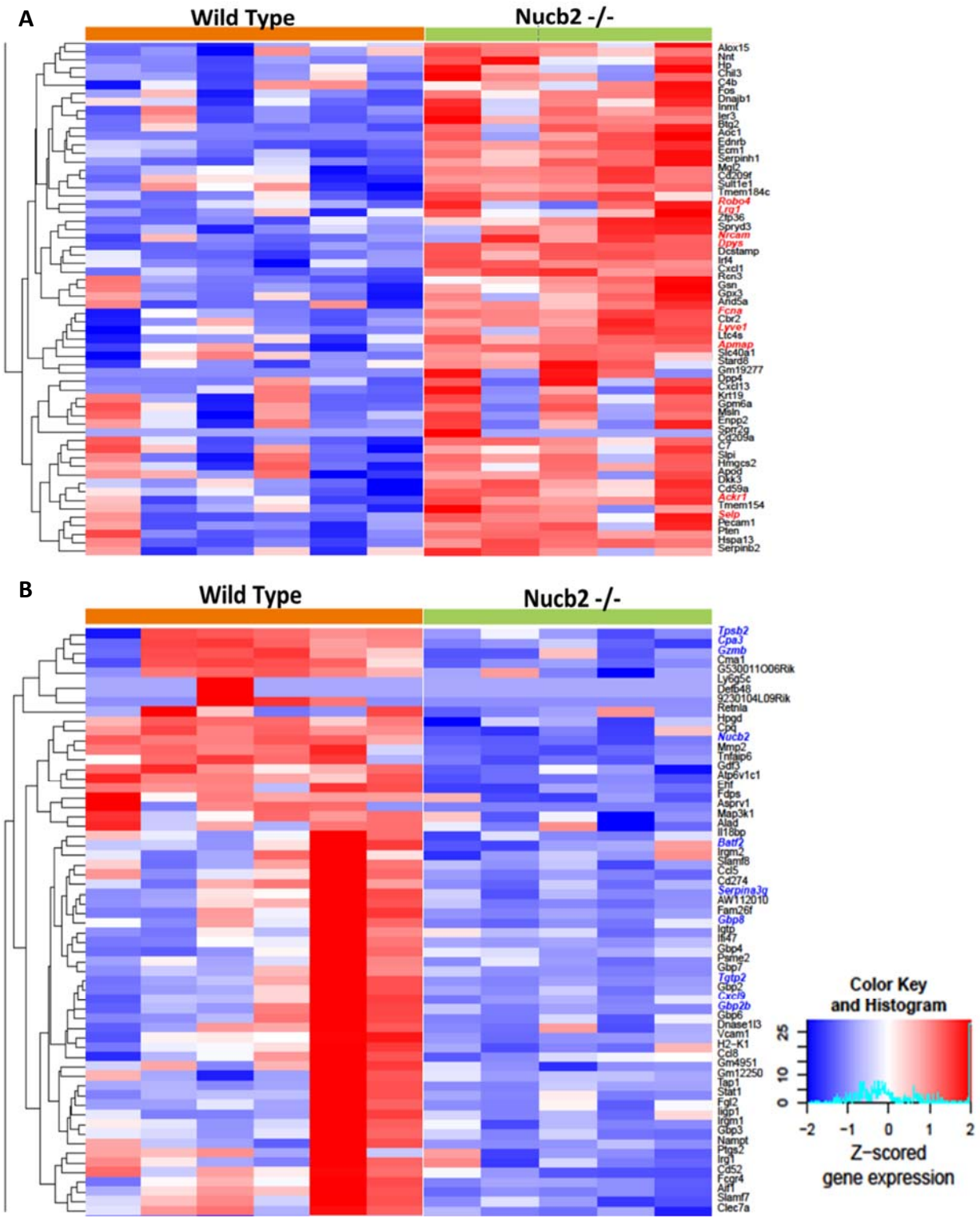


Figure 3.11: *Nucb2* ablation alters the transcriptome of adipose tissue macrophages

Heat map displaying unsupervised hierarchical clustering of normalized data (A) 58 significantly up regulated genes and (B) 64 downregulated genes. WT n=6, *Nucb2-/-* n=5

A

Major Upregulated Pathway

Pathway Name	Enrichment Score	Enrichment P-value
Tryptophan metabolism	4.00687	0.02
TGF-beta signaling pathway	3.97891	0.02
Valine, leucine and isoleucine degradation	3.66931	0.03
Glycolysis/Gluconeogenesis	3.2023	0.04

B

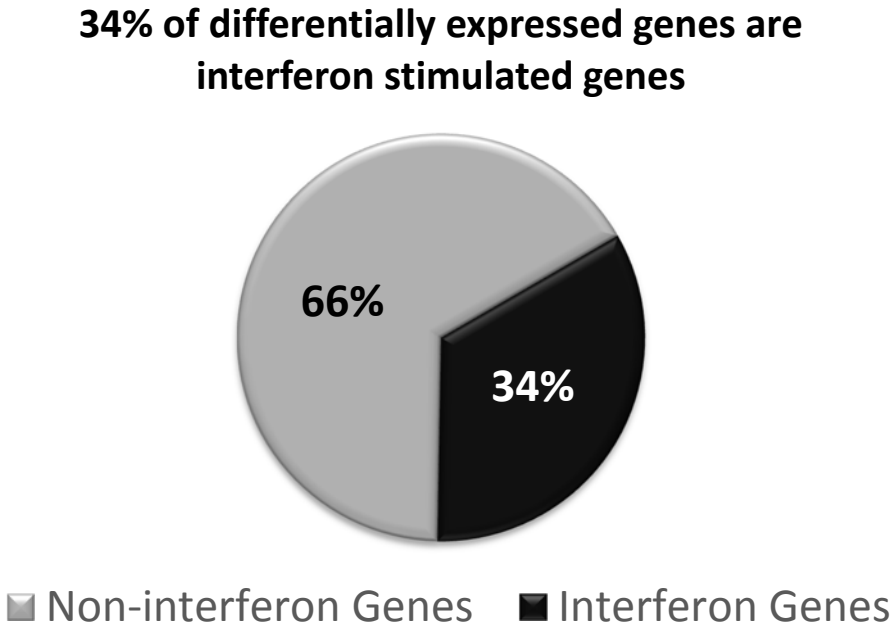


Figure 3.12: *Nucb2* ablation alters adipose tissue macrophage transcriptome of genes important for metabolic pathways

Using pathway analysis to uncover a collection of potential molecular interactions and reaction networks (A) major upregulated pathways in macrophages from *Nucb2-/-* mice and (B) Percentage of genes involved in type I interferon responses

These data further elucidate/validate the mechanism by which *Nucb2* is regulating immune cells. It is exciting that some of the major upregulated pathways from our RNA-sequencing is involved in the metabolism of M1 like proinflammatory macrophages. These data further prove that ablation of *Nucb2* renders macrophages to being more activated to a proinflammatory state. This proinflammatory state could be resulting in the insulin resistance phenotype seen in *Nucb2-/-* mice.

3.3.7 Insulin resistance and glucose disposal derangements are driven by myeloid specific *Nucb2* ablation but not *Nucb2* ablation in adipocytes

As shown above, *Nucb2* is important in regulating macrophage activation and inflammatory cytokine production. These data, in combination with the clear insulin resistance found in the *Nucb2-/-* mice, led us to investigate the mechanisms underlying this phenotype. We can think about two opposite cause and effect relationships: 1) Is *Nucb2* important in regulating the immune cells, and the ablation of *Nucb2* leads to increased cytokine production, thus resulting in insulin resistance; or 2) is *Nucb2* important for regulating insulin sensitivity in metabolic tissues directly, independent of the effects on immune cells, which in turn could potentially activate immune cells? These questions led us to produce tissue-specific, conditional *Nucb2* knockouts.

We therefore crossed our floxed Nucb2 C57Bl/6 mouse with Cre recombinase from the lysozyme M-encoding locus specific LysM^{cre} driver or adiponectin specific adipoq-^{Cre} to remove the floxed sequence exclusively in either innate myeloid cells or in adipocytes, respectively. We then carried out a glucose tolerance test (GTT) and an insulin tolerance test (ITT) in mice fed a HFD to determine whether *Nucb2* ablation, specifically in adipocytes or in myeloid specific cells, was sufficient to affect glucose homeostasis via insulin resistance.

Initially, we saw no differences in food intake or body weight between the wild type control, the Nucb2 adipocyte specific knockout and the *Nucb2* myeloid specific knockout in both chow and HFD conditions (Figure 3.13). Strikingly, we observed that the myeloid specific *Nucb2 -/-* mice had significant impairments in insulin sensitivity and impaired glucose tolerance. GTT revealed that blood glucose levels after glucose injection were significantly higher in LysM^{cre} *Nucb2-/-* mice than those in wild type and adipoq-^{Cre} *Nucb2-/-* animals. We next performed ITT (0.8 units/kg of body weight) in these mouse models to determine insulin sensitivity. Myeloid specific *Nucb2-/-* mice showed a hyperglycemic response to insulin compared to wild-type littermates as well as adipoq-^{Cre} *Nucb2-/-* animals.

Together, these data show that ablation of *Nucb2* specifically in myeloid cells is sufficient to replicate the severe insulin resistance phenotype seen in the global *Nucb2-/-*. This suggests that *Nucb2* in myeloid cells is responsible for the observed physiological impairments which indirectly lead to significant whole body insulin sensitivity.

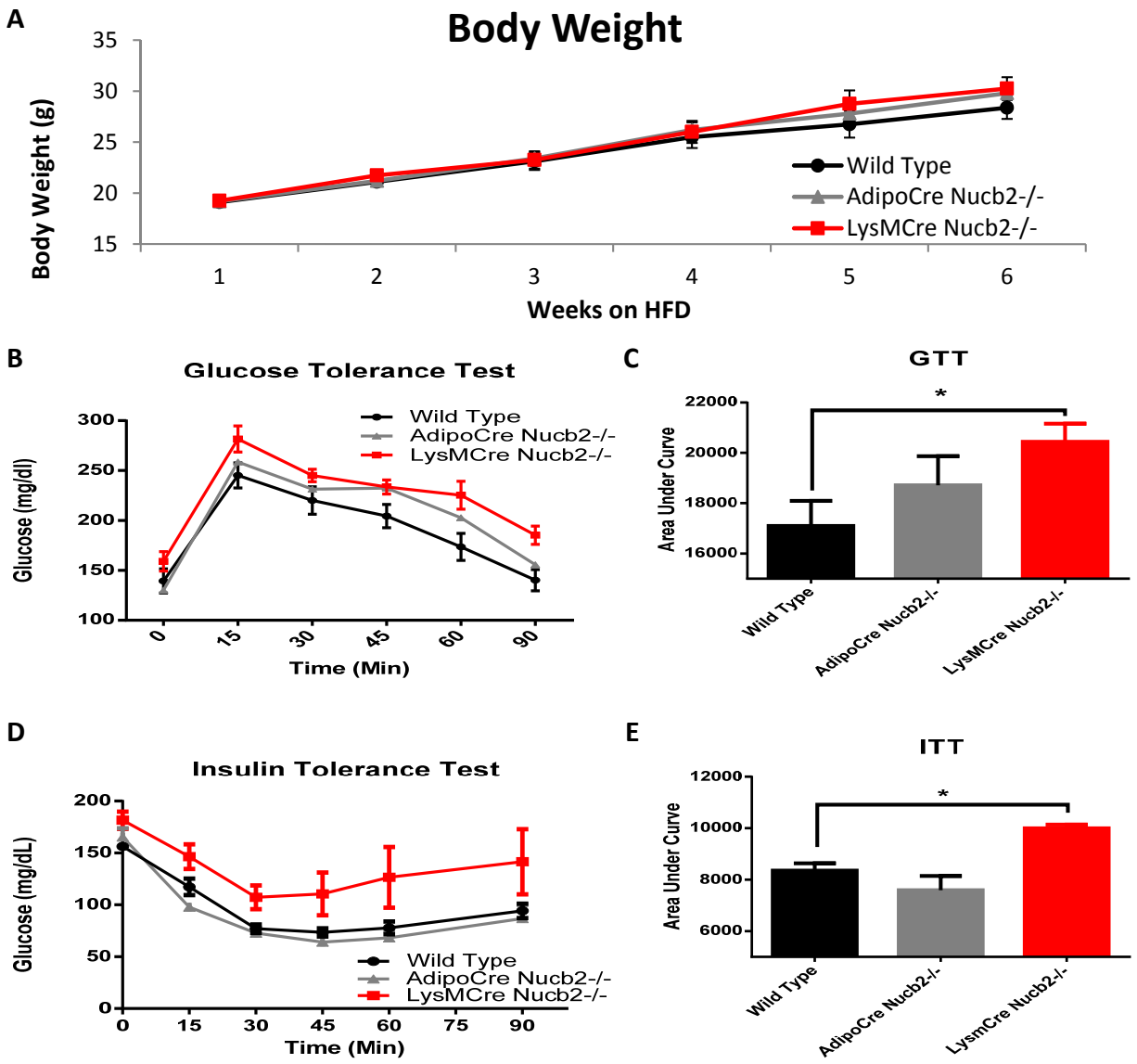


Figure 3.13: *Nucb2* ablation in myeloid cells increases insulin resistance and impairs glucose homeostasis

(A) Body weights of animals fed a HFD were measured weekly from 6 to 12 weeks of age. GTTs and ITTs in *Nucb2* specific knockout mouse models and blood samples from the tail were analyzed for glucose concentration. (B) For GTT, mice that were fed a high-fat diet were overnight-fasted then given an i.p. injection of 10% glucose (4 ul/g of body weight) and (C) expression by area under the curve. (D) For ITT, mice on high-fat diet were fasted for 4 h before i.p. administration of insulin, 0.8 units/kg of body weight and (E) expressed by area under the curve. Each point represents mean ± SEM; n = 5 per group. *P < 0.05.

Overall, these data illustrate that *Nucb2* in myeloid cells causes physiological changes within macrophages, which in turn indirectly affect systemic insulin sensitivity and induce a whole body insulin resistance state. Taken together with the previous results, *Nucb2* is an important regulator of proinflammatory cytokine production in response to a high fat diet. Without

regulation of immune cell proinflammatory cytokine production via *Nucb2*, mice are constitutively in an inflamed state which can lead to insulin resistance and impaired glucose uptake.

3.3.8 Macrophage ablation of *Nucb2* skews the transcriptome of adipose tissue macrophages towards a proinflammatory M1-like phenotype

To study how *Nucb2* is important in modulating other potential immune pathways, we tested adipose tissue macrophage transcriptome from *LysM^{cre}Nucb2-/-*. Furthermore, by comparing the global *Nucb2-/-* macrophage transcriptome to the macrophage specific *Nucb2-/-* transcriptome would allow us to further delineate potential intrinsic vs extrinsic effects of *Nucb2.*

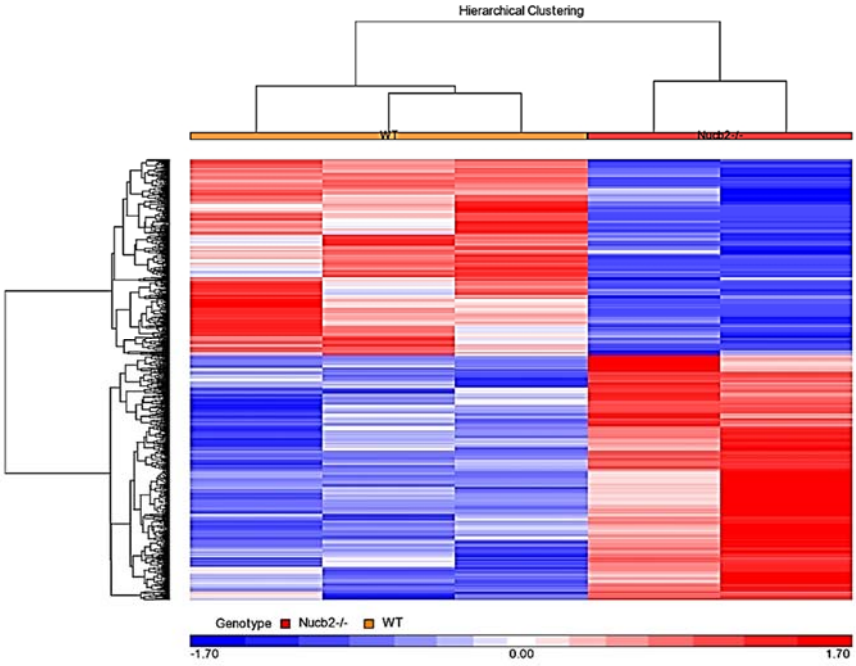
Similarly to macrophages isolated from the global *Nucb2-/-* mice, macrophages from LysM^{cre} *Nucb2-/-* mice also showed interesting changes in their transcriptome. In total, our RNA-sequencing from the LysM^{cre} *Nucb2-/-* mice expressed a difference in 469 genes when compared to wild type controls, 222 upregulated and 247 downregulated (Figure 3.14). Some of the genes that were differentially up or down regulated in the myeloid conditional *Nucb2-/-* mimicked those of the global *Nucb2-/-* mice (Figure 3.16). Of major interest to us is serum amyloid A 3 (SAA3). This is a potent proinflammatory protein that has been shown to be elevated in plasma of diabetic mouse models as well as human studies [168-170].

Under further investigation, our analysis revealed interesting pathways that are either up or down regulated. Our major upregulated pathways are involved in inflammatory responses and are transcriptomes of more M1-like macrophages. Not unexpectedly, TNF signaling pathway was the most upregulated pathway. Other pathways that were up regulated were not as expected; however they are in line with our increase in proinflammatory cytokine production by macrophages lacking the *Nucb2* gene. For example, HIF-1 signaling pathway was shown to be increased in our macrophages from LysM^{cre} *Nucb2-/-* mice. HIF-1 is important in mediating transcriptional activation of IL-1β [171, 172]. Furthermore, the upregulation of the mTOR signaling pathway also is known to be required for proinflammatory responses to several disease states [173, 174].

In concordance to these up regulated pathways being more proinflammatory, some of our major down regulated pathways are more anti-inflammatory. Although Mitogen-activated protein kinase (MAPK) can mediate proinflammatory processes, more recent studies suggest that MAPK has more important roles in mediating anti-inflammatory mechanisms [175, 176]. Our data suggests a significant downregulation of MAPK in macrophages lacking *Nucb2* gene. Furthermore, our data suggests a decrease in the toxoplasmosis pathway. This is significant because macrophages infected with toxoplasma strains are polarized towards an M2-like activation state and secrete anti-inflammatory molecules that can down-regulate Th1 immune processes. This process is important in the immune response against worm infections. It is

unclear how or even if *Nucb2* is working through this pathway. However, this gives us more evidence that macrophages lacking *Nucb2* are in a chronic proinflammatory state and even show decreases in anti-inflammatory states.

A



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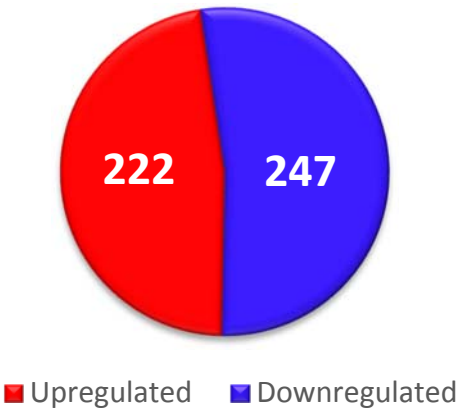

Figure 3.14: *Nucb2* ablation in macrophages alters adipose tissue macrophage transcriptional signatures

(A) Heat map displaying the hierarchical clustering of the 1000 most differentially expressed genes (B) Number of differentially expressed genes with at least a 2 fold increase or decrease. WT n=3, LysMcre *Nucb2-/-* n=2

A

Major Upregulated Pathways

Pathway Name	Enrichment Score	Enrichment P-value
TNF signaling pathway	4.99217	0.007
HIF-1 signaling pathway	3.51362	0.03
mTOR signaling pathway	3.51037	0.03

B

Major Downregulated Pathways

Pathway Name	Enrichment Score	Enrichment P-value
MAPK Signaling pathway	9.5822	0.00006
Jak-STAT signaling	4.40386	0.01
Toxoplasmosis	2.98034	0.05

Figure 3.15: *Nucb2* ablation in macrophages alters the adipose tissue macrophage transcriptome of metabolic and inflammatory pathways

Using pathway analysis to uncover a collection of potential molecular interactions and reaction networks (A) major upregulated pathways and (B) major downregulated pathways

Overall, these data overlap in adipose tissue macrophage transcriptome differences between global *Nucb2-/-* and LysM^{cre} *Nucb2-/- mice*. These similarities suggest that *Nucb2* is playing an intrinsic role in regulating the inflammatory response in macrophages. Furthermore, the pathway analysis gives us informative insight in to other potential innate immune pathways that might be modulated or mediated by *Nucb2* which contribute to an overall insulin resistance phenotype. This data clearly show that our macrophages lacking *Nucb2* are more proinflammatory M1-like macrophages.

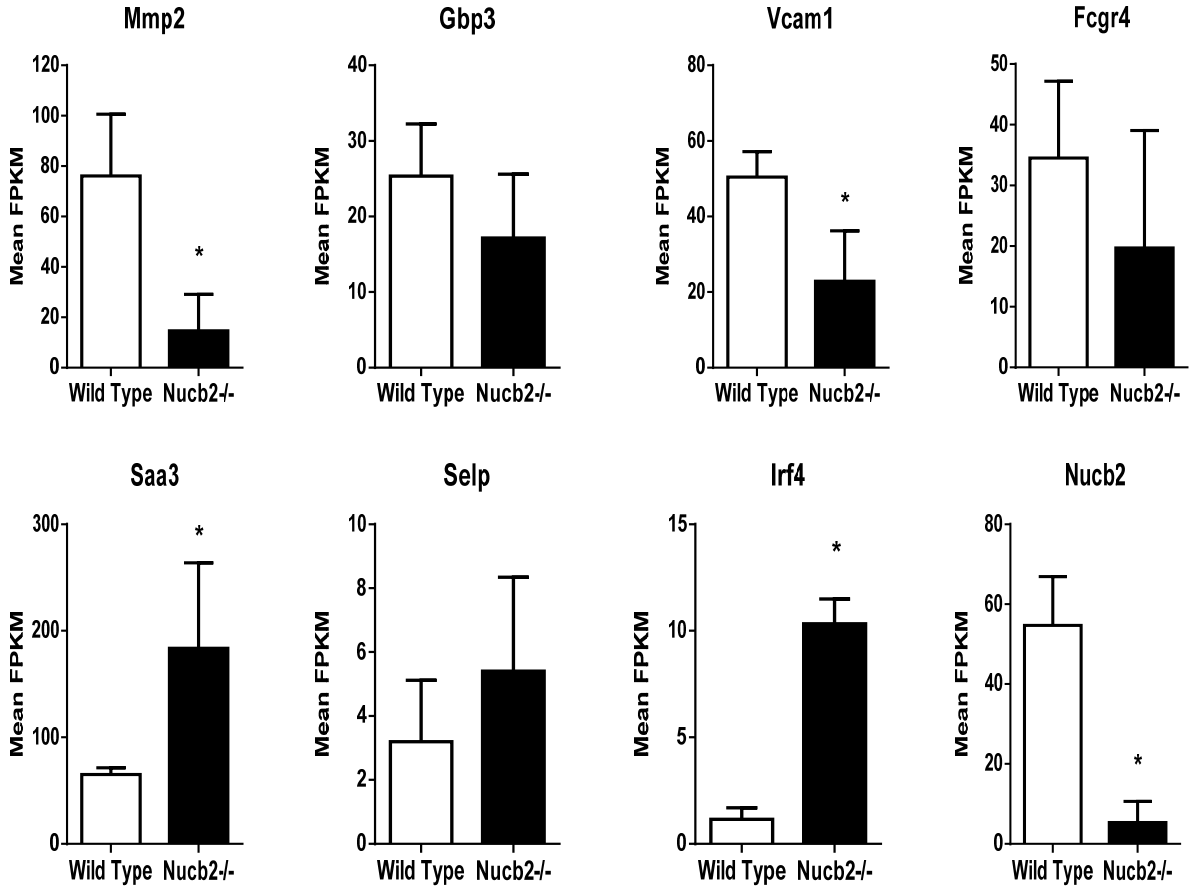

Figure 3.16: *Nucb2* ablation in macrophages alters the inflammatory transcriptome

From our RNA-sequencing, we determined several overlapping ISG expression levels in macrophages either from global *Nucb2-/-* mice or LysM^{cre} Nucb2-/- mice. Differentially expressed genes that overlap between LysM^{cre} Nucb2-/- macrophages and macrophages from global Nucb2-/- mice. WT n=3, LysMcre *Nucb2-/-* n=2

3.4 Discussion

The major findings of these experiments show that **1)** *Nucb2* is not very important for immune cell chemotaxis, however, it is an important regulator of M1-like classically activated proinflammatory macrophages and important for regulating the production of proinflammatory cytokines as shown by *in vitro* BMDM model and *in vivo* sorted macrophages. **2)** *Nucb2* normally regulates an inflammatory cascade by down regulating NFκB's phosphorylation and its translocation to the nucleus. **3)** *Nucb2* is critical in mediating type I interferon response, as shown by significant differentially expressed Interferon-stimulated response genes in macrophages from wild type vs Nucb2 KO animals. **4)** *Nucb2* ablation in myeloid lineage cells is sufficient to cause both decreases in insulin sensitivity and whole body glucose homeostatic impairments.

These data uncovered the significance of *Nucb2* in the regulation of proinflammatory cytokine production. Not only is *Nucb2* important in inflammation, the unbiased transcriptomic profiling using RNA sequencing suggested that NucB2 deficiency impairs the type I interferon responses. It is interesting and important to note, however, that these responses are not in an infectious model, however occurring in a sterile, metabolic challenge with a high fat diet induced obesity model. We found that *Nucb2* is mediating important regulatory functions of NFκB, and that this regulation maintains basal levels of proinflammatory states.

Nucb2 expression increases in macrophages and T cells under conditions of high fat feeding. This increase in *Nucb2* levels may be a compensatory mechanism to decrease chronic-systemic inflammation in adipose tissue derived immune cells. High fat diet has been shown to be associated with increased inflammation and inflammatory cytokine production [20, 22, 92, 158] as well as increased nesfatin-1 levels [142, 144]. It is therefore plausible that macrophages and T cells respond by increasing production of *Nucb2* to try to dampen this inflammatory response. It would also be intriguing to determine if *Nucb2* is dysregulated or even becomes resistant during chronic high fat diet and severe obesity related type 2 diabetes. In fact, one study implied through correlation studies in cerebrospinal fluid of obese and lean individuals that nesfatin-1/NUCB-2 has protein binding capabilities, and that differences in protein binding may be a result of resistance. Finally, the authors show that the efficiency of nesfatin-1/NUCB-2 uptake into CSF is reduced in obese individuals, possibly due to saturation of transporters [177], however they do mention that further research is necessary. These data are intriguing, however, these data are potentially spurious due to the unreliable commercially available ELISA kits that were used in these studies.

Such dysregulation of NFκB in *Nucb2 -/-* mice could further exacerbate the sterile inflammatory state in metabolic tissues which in turn renders cells insulin resistant. Interestingly, other studies have identified that *Nucb2* expression correlates positively with plasma levels of IL-6 and TNF-α in patients with COPD. The authors of this study concluded that *Nucb2* is a novel inflammatory factor in stable emphysematous COPD, however they did not show a causal relationship between the two [178]. The authors could have erroneously concluded that such positive correlation may reflect a proinflammatory connection. Our data strongly suggests that *Nucb2* is positively correlated with proinflammatory cytokine production in an attempt to decrease inflammatory cytokine production via inhibition of NFκB transcription.

Other studies have shown that *Nucb2* mRNA and nesfatin-1 protein levels are significantly higher in high-fat fed mice (vs chow diet) and are reduced under food deprivation [134]. They further showed that food deprivation decreases *Nucb2* expression in subcutaneous adipose tissue and that stimulation of subcutaneous adipose tissue explants with TNF-α, IL-6, insulin and dexamethasone significantly increases intracellular nesfatin-1 levels. Due to non-specificity of

commercially available reagents, however, these studies with rigorous measures of nesfatin-1 protein levels or effects of nesfatin-1 on cell types cannot be interpreted correctly.

Originally, adipose-tissue-derived peptides (cytokines and adipokines) were thought to regulate energy metabolism and to be associated with the chronic low-grade inflammation present in obesity-related metabolic disturbances and inflammatory diseases. In myeloid specific *Nucb2* knockout mice, the inability to regulate macrophage induced-inflammation was sufficient to cause a diabetic phenotype. Together, our data indicate that *Nucb2* is an important regulator of proinflammatory cytokine production in an NFκB dependent mechanism. Indeed, *Nucb2* regulates NFκB activity in cells, though our data do not fully pinpoint at which stage *Nucb2* regulates NFκB. We, did, however show that inhibiting the translocation of NFκB reversed all the inflammatory phenotype seen in macrophages lacking *Nucb2*. NFκB is usually present in cells in an inactive state and does not require new protein synthesis in order to become activated. In fact, in unstimulated cells, NFκB is sequestered in the cytoplasm by a family of inhibitors, IkBs which become degraded upon cell activation through signaling components. This allows free NFκB to enter the nucleus and modulates the transcription of many proinflammatory genes. It is plausible that *Nucb2* acts on IkB proteins to maintain NFκB inhibition. However more studies are required to determine whether upstream pathways that control *Nucb2*-NFκB interactions.

The RNA sequencing from isolated adipose tissue immune cells has given us many important insights into the molecular mechanisms by which *Nucb2* regulates inflammation and insulin sensitivity. Interestingly, the majority of the genes that are differentially regulated in macrophages from *Nucb2-/-* mice are those of type I interferon response. As well as ISGs, some of the major pathways that were elucidated were those of inflammatory M1 like macrophage metabolism. For example, glycolysis and tryptophan metabolism pathways were both up regulated in the adipose tissue macrophages from our *Nucb2-/-* mice. Individual genes of great interest were the increases in Dipeptidyl-peptidase 4 (DPP4) and the increases in acute-phase SAA3 levels in our *Nucb2-/-* mice, both of which have been strongly implicated in glucose metabolism and the development of type 2 diabetes.

To further elucidate the mechanisms by which *Nucb2* impact glucose homeostasis, we generated both adipocyte specific and myeloid specific *Nucb2* knockout mice. Our findings proved that the deleterious effects on insulin resistance is driven mostly by the lack of *Nucb2* in myeloid cells, and not in adipocytes. Following these findings, we investigated functional differences in adipose tissue isolated macrophages to determine the cause of the metabolic dysfunction associated with the deletion of *Nucb2 (-/-)*. We therefore performed genome-wide comparisons of the transcriptomes of adipose tissue macrophages from wild type and *Nucb2-/-* mice. Our RNA-sequencing suggested that *Nucb2* is important in regulating type I interferon response genes, although this has not been rigorously tested.

DPP4 is a glycoprotein that is ubiquitously expressed (lung, brain, pancreas, kidney, blood vessels, thymus, lymph nodes and spleen) on the surface of a variety of cells including epithelial, endothelial and immune cells such as lymphocytes and monocytes [179, 180]. Interestingly, DPP4 can also be cleaved from the membrane and released into the circulation by a process called shedding [181, 182]. Notably, DPP4 expression is dysregulated in a variety of disease states including chronic inflammation, cancer, obesity and diabetes. Since DPP4 has been characterized as an adipokine which correlates with the magnitude of the severity of the metabolic syndrome, it can be used as a reliable molecular biomarker [183, 184] for this syndrome. Interestingly, monocytes express high levels of DPP4, and these levels are further upregulated in proinflammatory states, including those linked to obesity and T2D. Furthermore, a competitive DPP4 inhibitor, sitagliptin, has been shown to have potent systemic anti-inflammatory properties, as it suppresses expression of proinflammatory genes in mice and humans and locally in adipose tissue of obese individuals [185, 186].

Interestingly, our data shows a significant increase of DPP4 expression in adipose tissue macrophages lacking *Nucb2.* It is likely that this increase in DPP4 contributes to the overall inflamed state of the adipose tissue in our *Nucb2-/-* mice, as well as their insulin resistant phenotype, via DPP4 mediated activation of NFκB. Furthermore, DPP4 in both its soluble or membrane bound form, is able to interact with caveolin-1, which leads to a downstream activation of NFκB via IRAK-1 dependent mechanisms [187, 188]. Finally, DPP4 has been shown to inactivate the incretin hormone glucagon-like peptide (GLP-1) in the periphery and that DPP4 inhibition increases glucose uptake [189, 190]. Overall, this link with high DPP4 in *Nucb2 -/-* macrophages may affect the bioavailability of its substrates GLP1, which would further decrease glucose uptake. These data further elucidate a potential mechanism by which *Nucb2* is down regulating adipose tissue macrophage expression of DPP4 which in turn improves glucose uptake.

Another interesting gene target identified from our RNA-sequencing study that may link *Nucb2* to insulin-resistance is SAA. Host response to injury and infection is accompanied by a rapid rise of blood acute-phase proteins, including SAA [191, 192]. Clinically, increases in SAA have been widely used as an important indicator in the diagnosis and prognosis of inflammatory diseases [193-195]. Furthermore, T2D has been shown to be preceded by an acute-phase SAA secretion, a component of the innate immune system and a sensitive marker for acute inflammatory state [168, 169]. SAA exhibits significant immunological activity by inducing secretion and activation of many cytokines (IL-1β, TNF, MIP-1α and IL-6) in macrophages and lymphocytes, as well as by acting as a chemotactic factor for leukocytes [196-198]. Intriguingly, SAA3 was the most upregulated gene in our *Nucb2-/-* isolated adipose tissue derived macrophages, as compared to wild type macrophages. It is plausible that *Nucb2* is functioning either upstream or downstream of SAA and is necessary to regulate the secretion of many proinflammatory cytokines.

Macrophages are cells that are highly regulated by both internal and external signals. To determine the cell intrinsic vs extrinsic effects of *Nucb2* on macrophages, we performed RNA-sequencing in the macrophages from myeloid LysM^{cre} *Nucb2-/-*. Our hypothesis was that macrophage intrinsic regulation of *Nucb2* is responsible for the regulation of inflammatory cytokine production, which indirectly disrupts insulin sensitivity. Most importantly, ablation of *Nucb2* in myeloid lineage alone is sufficient to render mice insulin resistant. This was evident by LysM^{cre} *Nucb2-/-* impairments in insulin tolerance and glucose tolerance test. This shows that macrophage specific *Nucb2* expression is necessary for regulation of obesity related inflammation and is required for proper maintenance of glucose homeostasis.

Interestingly, we saw many similarities between the global and conditional *Nucb2-/-* macrophage transcriptome, however differences also arose. It is plausible that the similarities between the global knockout and myeloid conditional knockout exist from the intrinsic effects of *Nucb2.* Furthermore, it is plausible that the differences in transcriptomes between these groups arise from external signals where *Nucb2* is affecting different cells, and these cells signal to the macrophages differently. For example, the up or down regulated genes in the macrophages from the global *Nucb2-/-* mice, might be receiving a signal (cytokine) from a T cell which also does not express *Nucb2,* whereas the conditional *LysM^{cre} Nucb2-/-* mice receives a different signal since those T cells do express the *Nucb2* gene. Further studies are required to differentiate the intrinsic vs. extrinsic effects of *Nucb2* on macrophages.

From all our studies, it is clear that *Nucb2* is an important regulator of different facets of the immune-metabolic axis. Of major interest were the findings that *Nucb2* is required for proper regulation of obesity induced inflammation and insulin resistance. Moreover, our RNA-sequencing further implicates a novel role of *Nucb2* in type I interferon response. From these results, however, more questions are needed to be answered 1) whether type I interferons controls metabolism directly? 2) Whether *Nucb2* plays a direct role in mediating IFN effects on metabolism? 3) Whether *Nucb2* expression in innate immune cells is required for protection against viruses?

Although we have studied at *Nucb2* during responses to different metabolic conditions, it remains important to determine the effect of *Nucb2* during pathogen-induced immune responses. To do so, it would be necessary to use our experimental model to determine the role of *Nucb2* in a typical inflammatory response such as lipopolysaccharide induced endotoxemic challenge. Furthermore, because of the impact *Nucb2* has on the interferon response, it is also imperative to challenge these mice with different viral or bacterial infections (see Ch. 4).

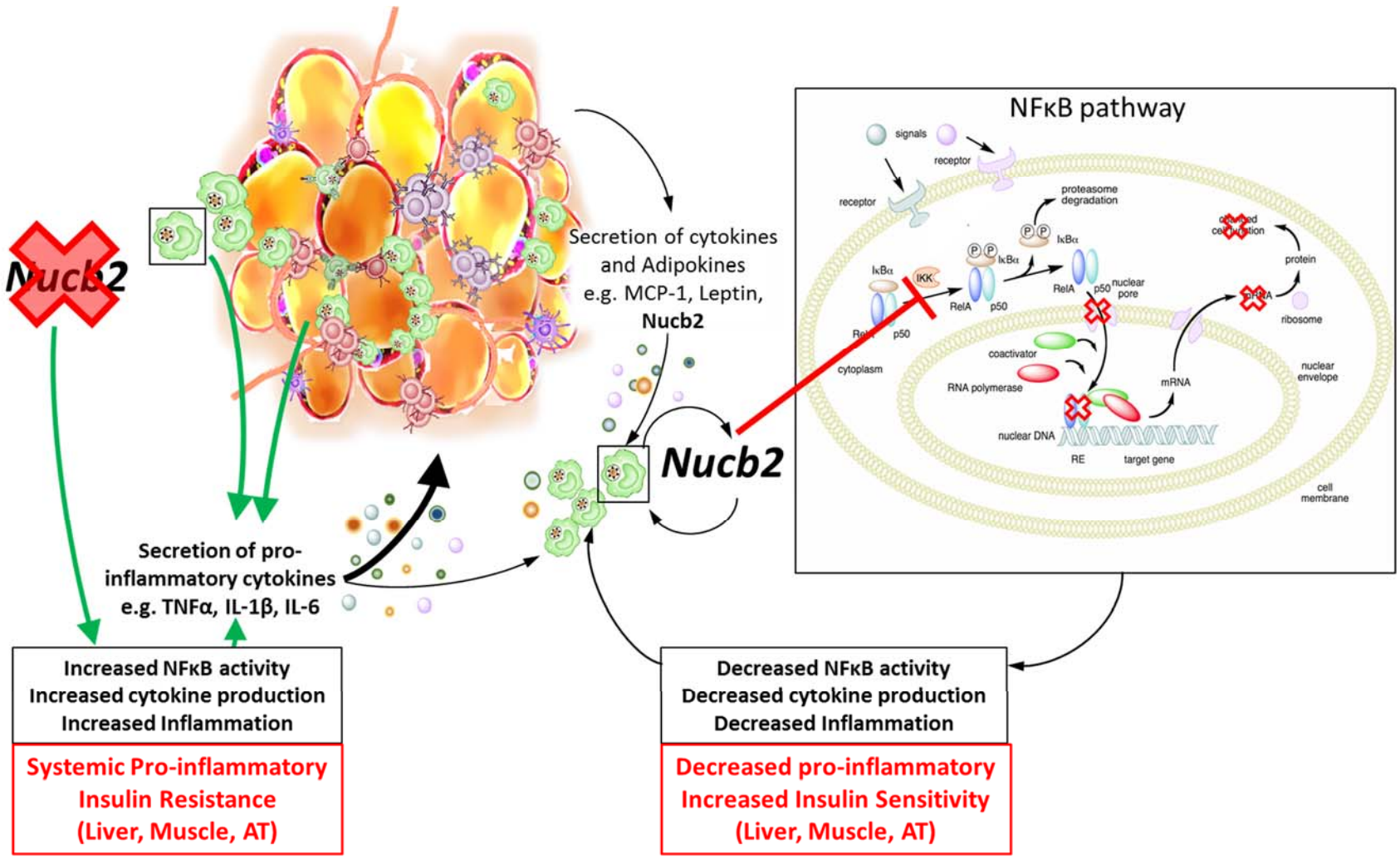

Figure 3.17: Schematic of how *Nucb2* regulates obesity induced inflammation through NFκB pathway

On the left, we are showing what is occurring when we remove *Nucb2* from macrophages. The box on the right is the NFκB pathway whereby *Nucb2* is acting to reduce the activity of NFκB which further diminishes the transcription of proinflammatory cytokines.

Chapter 4: Regulation of Lipopolysaccharide (LPS)-induced Inflammation and endotoxemia by Nucleobindin-2

4.1 Introduction

Systemic inflammation, resulting from both infections and noninfectious conditions, is a major cause of morbidity [199, 200]. It can arise from bacterial infection and cause multiple organ dysfunctions, or can be a result of an external stimulus such as severe burns. Both are different in nature, however both lead to a dramatic immune response with elevated levels of inflammation. These responses can result in a cytokine storm with sepsis, which can lead to septic shock and death. In light of our previous findings that *Nucb2* affects the inflammatory sterile response to obesity, we wanted to see how ablation of *Nucb2* may affect the immune response to well-studied endotoxemia.

Endotoxin is the cell wall component of Gram-negative bacteria, and plays a central role in the pathogenesis of septic shock. In humans, an injection of small doses of endotoxin (4 ng/kg) triggers acute systemic inflammatory responses which are qualitatively similar to those that occur during the onset of septic shock. Indeed, i.v. injection causes direct release of cytokines, which subsequently cause fever and leukopenia followed by leukocytosis and shock. Shortly after administration, changes occur in systemic homeostatic processes in many different organs. In concurrence with organ functional changes, inflammatory mediators are released and contribute to the responses to eradicate the microbe. These include, but are not limited to, the proinflammatory cytokines TNF-α, IL-1β, IL-6 and IL-18, as well as chemotactic cytokines MIP-1α and MIP-1β. There are many major homeostatic pathophysiological derangements during severe sepsis. Among the changes, hyperglycemia seems to be of most importance. Hyperglycemia arises from insulin resistance as well as increased muscle glycolysis and lipolysis with subsequent gluconeogenesis and glycolysis in the liver.

A classic murine model to study systemic inflammation, sepsis and septic shock is the LPS-induced Toll-like receptor 4 dependent systemic inflammation. Although these models are useful to understand mechanisms, they are somewhat different from the human situation. First, humans are as much as 100,000-fold more likely to succumb to an intravenous dose of LPS than mice. An LPS concentration of 1 ug/kg in humans is enough to induce septic shock, whereas mice require significantly higher concentrations [201]. For studying the onset of systemic inflammation and the acute innate immune response, murine i.p. injections are good to induce rapid entry of LPS and acute phase reactions [202]. This is a valuable model to investigate key systemic responses involving the sympathetic-adrenomedullary axis, acute phase protein production, inflammation, thermoregulation and alterations in leukocyte responsiveness to endotoxin agonists prior to the onset of bacteremia (toxicity of these bacteria). It is important to note that

endotoxin sepsis, organ failure and ultimately death does not occur as a result of a bacterial infection, however it ensues by an exaggerated immune response.

Up to now, our findings have underlined the importance of *Nucb2* in the maintenance of the inflammatory processes in both LPS induced BMDMs as well as non-infected responses to high fat feeding. Therefore, in the present study, we set out to investigate whether *NUCB2* is important *in-vivo* for the acute inflammatory cytokine response in blood leukocytes in response to an LPS-induced endotoxemic shock. Injection of LPS into experimental animals is known to trigger massive production of TNF-α, IL-1β as well as other proinflammatory cytokines which cause sickness behavior. We examined typical metabolic responses to infection, including core body temperature, circulating glucose levels, and body weight. We also examined the circulating levels of typical proinflammatory cytokines in response to LPS-stimulated endotoxic shock. Finally, we examined the capacity of spleen leukocytes to respond to whole body bacteria after *in vivo* exposure to LPS. Our study aimed to test if *Nucb2* is required to mount a proper response to an *in vivo* endotoxin LPS exposure.

4.2 Materials and Methods

4.2.1 Mice and animal care

Mice were kept in a pathogen-free barrier facility maintained at 22–24°C with a 12:12-h dark-light cycle (lights on at 0700 h). Mice were housed up to 5 per cage and given *ad-libitum* access to normal chow (at least 4.5% kcal crude fat) and sterilized water (hydropac Alternative Watering System: Seaford, DE, USA). All transgenic and WT mice in our colony were cross-fostered to parent cohorts in our colony. The sentinel mice in our animal rooms were negative for tested standard murine pathogens. Those included ectromelia, epizootic diarrhea of infant mice [EDIM], lymphocytic choriomeningitis [LCMV], Mycoplasma pulmonis, mouse hepatitis virus [MHV], murine norovirus [MNV], mouse parvovirus [MPV], murine minute virus [MVM], pneumonia virus of mice [PVM], reovirus type 3 [REO3], Theiler's murine encephalomyelitis virus [TMEV], and Sendai virus. These pathogens were tested at various times during the course of the studies. Chronological changes in body weights were measured during the life of the animals.

4.2.2 Experimental design and LPS injection

Mice were aged to 14-16 weeks before beginning the study. Mice were subjected to intraperitoneal injection (i.p.) of 2 mg/kg of LPS from *Escherichia coli* serotype (L3024; Sigma–Aldrich, St Louis, MO, USA) dissolved in saline solution. In the control group, animals received an injection with sterile saline solution (NaCl 0.9%). Metabolic measurements were performed at baseline, 2 hrs and 4 hrs post i.p. injection. Four hours after administration of LPS or saline, the animals were euthanized with isoflurane and decapitated. All experiments were performed on

male mice and done at 2hrs and 4 hrs in wild type with saline (n=3 per time point) and LPS (n=3), as well as in *Nucb2* -/- mice with LPS (n=3). All data is combined for an n=6 in each of the three groups.

4.2.3 Body temperature and blood glucose measurements

Core body temperature was measured using a rectal thermocouple thermometer Physitemp BAT-12 (Physitemp: Clifton, NJ, USA). Blood glucose was detected using Breeze2 blood glucose test strips (Bayer HealthCare; Mishawaka, IN, USA) via tail bleed. Measurements were taken at baseline, 2 hrs and 4 hrs post LPS/saline injections.

4.2.4 Quantitative real time PCR

RNA from tissue and cells were isolated using an RNeasy Plus mini and micro kit (Qiagen; 74106 and 74034) according to the manufacturer's instructions. DNA digestion was performed on the columns by following the manufacturer's instructions (79254; Qiagen). Following RNA purification, these samples were then used for iScript cDNA synthesis using a reverse transcriptase PCR kit (BIO-RAD; Hercules, CA, USA). Quantitative PCR was performed with the LightCycler 480 II (Roche Applied Science; Indianapolis, IN, USA) and Power SYBR Green detection reagent (Applied Biosystems by Thermo Fischer Scientific; Woolston Warrington, UK). Primer sequences for transcripts encoding proteins involved in lipid and glucose metabolism were designed with Primer Express Software. For *Nucb2*, we used the forward primer sequence 5'-AAAACCTTGGCCTGTCTGAA-3' and the reverse primer sequence 5'-CATCGATAGGAACAGCTTCCA-3'. For CCL4, we used the forward primer sequence 5’-GAAACAGCAGGAAGTGGGAG-3’ and the reverse primer sequence 5’- CATGAAGCTCTGCGTGTCTG-3’. For IL-1β, we used the forward primer sequence 5’-GGTCAAAGGTTTGGAAGCAG-3’ and the reverse primer sequence 5’-TGTGAAATGCCACCTTTTGA-3’. For IL-6, we used the forward primer sequence 5’-ACCAGAGGAAATTTTCAATAGGC-3’ and the reverse primer sequence 5’-TGATGCACTTGCAGAAAACA-3’. For TNF-α, we used the forward primer sequence 5’-AGGGTCTGGGCCATAGAACT-3’ and the reverse primer sequence 5’-CCACCACGCTCTTCTGTCTAC-3’. For MIP-1α, we used the forward primer sequence 5’-ACCATGACACTCTGCAACCA-3’ and the reverse primer sequence 5’-GTGGAATCTTCCGGCTGTAG-3’.

In all qRT-PCR experiments, 12.5ng cDNA was used. Fold induction of gene expression with *Nucb2* was analyzed with the ΔΔCt method (also known as the comparative Ct method) as determined by the following equation: ΔΔCt = ΔCt treatment (WT and *Nucb2* -/- LPS i.p.) - ΔCt control (WT control). Here, the ΔCt is the Ct value for the sample treatment normalized to the endogenous housekeeping gene GAPDH.

4.2.5 Immune profiling by Bio-Plex assay

Concentrations of immune cytokines in sera and BMDM supernatants were determined using mouse bio-plex pro Th17 cytokine assay (Bio-Plex Pro Array System, Bio-Rad, Hercules, CA,

USA), following the manufacturer's instructions. BMDM samples were directly assayed and serum samples were diluted 1:4 in sample diluent and incubated for 1 h (room temperature, 850 rpm agitation) with capture antibody-coupled magnetic beads. Following three washes in a Bio-Plex Pro wash station, samples were incubated for 30 min with biotinylated detection antibody in a dark (room temperature, 850 rpm agitation). Each captured analyte was detected by the addition of streptavidin-phycoerythrin and quantified using a BioPlex array reader. Analyte concentrations were calculated with Bio-Plex Manager software.

4.3 Results

4.3.1 LPS-induced endotoxemia significantly alters body temperature and glucose levels

Our previous findings showed strong implications that *Nucb2* negatively regulates metabolic inflammatory responses to obesity. It is thus important to determine if *Nucb2* is important in regulating pathogenic immune response, where inflammation is magnitudes higher than obesity-induced inflammation. To study the role of *Nucb2* on a more pathogen induced inflammatory response, we used LPS induced *Nucb2-/-* mice to determine its effects on systemic inflammation, sepsis and septic shock. To determine this inflammatory responses in the short term, we challenged WT and *Nucb2-/-* mice with high doses (2 mg/kg) of LPS from *Escherichia coli* via i.p. injections and followed them for 2 and 4 hrs.

At baseline, there were no differences in measured metabolic parameters in the *Nucb2-/-* mice compared to WT animals. At baseline, the body weights of WT mice were 22.83 ± 0.48g, 23.17 ± 0.17g in WT LPS and 22.83 ± 0.48g in *Nucb2* -/- (p=0.5). Also, there was no differences in glucose levels at baseline (WT saline: 134.5 ± 6.0 mg/dl, WT LPS: 144.3 ± 5.48 mg/dl and *Nucb2* -/-: 145.7 ± 5.94 mg/dl, p=0.2). Finally, there was also no difference in core body temperature between the three groups (WT saline: 37.95 ± 0.08 °C, WT LPS: 37.98 ± 0.05 °C and *Nucb2* -/-: 37.73 ± 0.1 °C, p=0.7).

Interestingly, there was a sharp, significant decrease in blood glucose levels in the short term response to LPS in both WT and Nucb2-/- mice. The significant decrease in glucose levels was present 2 hrs after LPS administration (WT saline: 120.0 ± 5.3 mg/dl, WT LPS: 104.3 ± 3.28 mg/dl and *Nucb2* -/- LPS: 95.2 ± 5.0 mg/dl, p=0.007). There was no difference in glucose levels between wild type LPS injected mice and *Nucb2* -/- LPS injected mice (p=0.2). The drop in glucose level was even more dramatic and maintained after 4 hrs (WT saline: 124.7 ± 2.1 mg/dl, WT LPS: 73.2 ± 6.0 mg/dl and *Nucb2* -/-: 64.8 ± 2.9 mg/dl, p<0.0001). In total, the wild type LPS had a drop of glucose levels of 71.2 ± 10.8 mg/dl and the *Nucb2* -/- LPS injected mice had a drop of glucose levels of 80.8 ± 5.0 mg/dl, p<0.0001.

In parallel to the decrease in glucose levels, there was also significant reductions in core body temperature in the short term response to LPS. 2 hrs post LPS injection decreased body temperatures in both WT and Nucb2 -/- animals (WT saline: 37.8 ± 0.03 °C, WT LPS: 36.72 ± 0.24 °C and *Nucb2* -/- LPS: 36.85 ± 0.04 °C, p=0.0001). There was no difference in core body temperature between wild type, LPS injected mice and *Nucb2* -/- LPS injected mice, p=0.6. This drop in body temperature was even more extreme after 4 hrs (WT saline: 37.43 ± 0.13 °C, WT LPS: 34.95 ± 0.47 °C and *Nucb2* -/-: 35.58 ± 0.10 °C, p<0.0001). In summary, LPS injection decreased body temperature of WT mice by 3.03 ± 0.44 °C and the *Nucb2* -/- mice by 2.15 ± 0.17 °C, p=<0.0001.

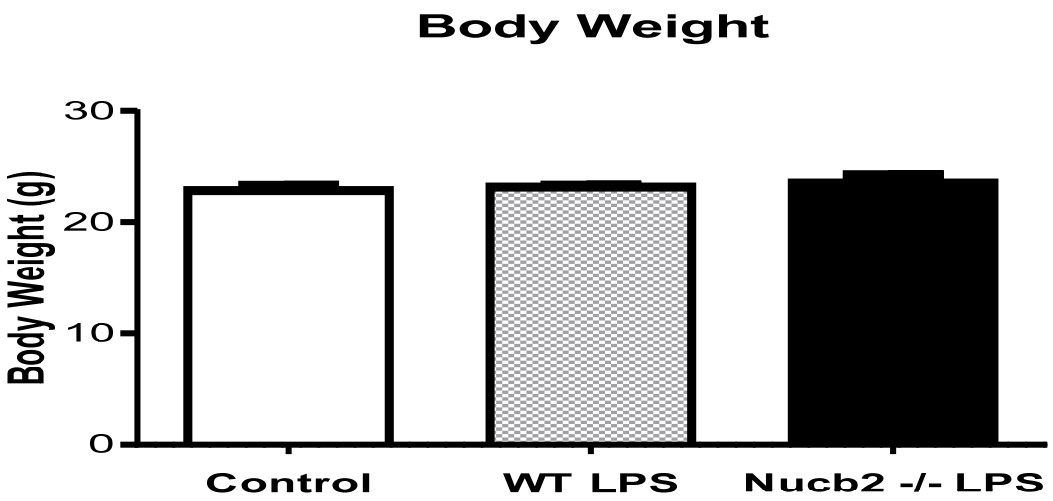

Figure 4.1: *Nucb2* ablation does not affect body weight

Body weights were measured prior to treatment and are represented in grams for wild type sham, wild type LPS injected and *Nucb2-/-* LPS injected, 14-16 week old mice. All data are represented as mean ± SEM (n=6 per group).

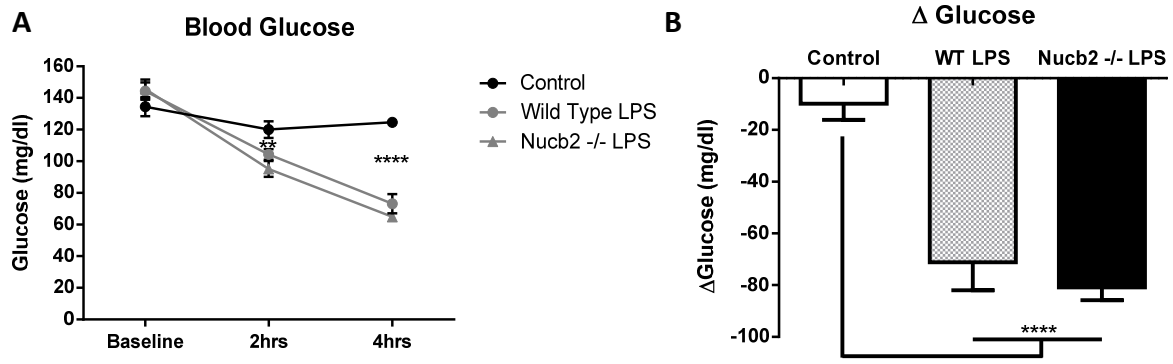

Figure 4.2: *Nucb2* does not alter glucose metabolism during an LPS challenge

Blood glucose levels were measured at baseline, 2 hrs and 4 hrs after injection and are represented as mg/dl. (A) Glucose levels over time and (B) change in glucose levels from baseline, pre injection glucose levels. All data are represented as mean ± SEM (n=6 per group). *p< 0.05

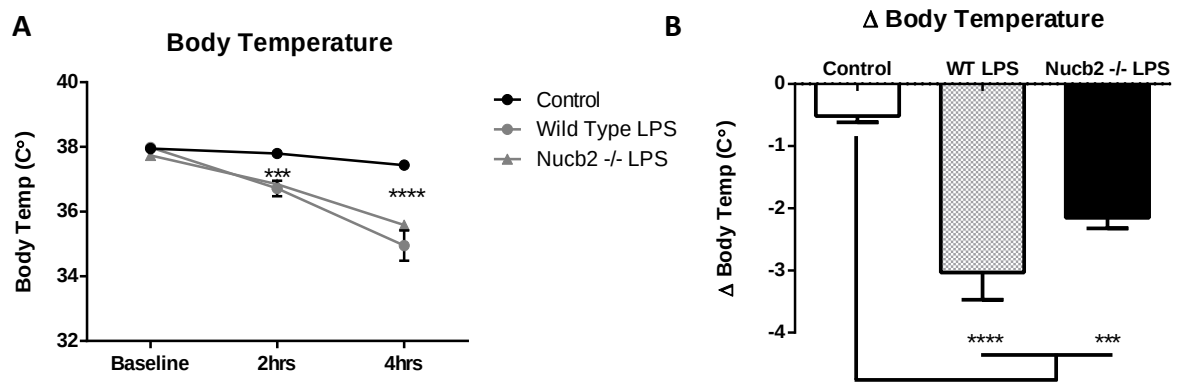

Figure 4.3: *Nucb2* does not alter hypothermic response to an LPS challenge

Body temperatures were measured at baseline, 2 hrs and 4 hrs after injection and are represented degrees Celsius. (A) Body temperature over time and (B) Change in body temperature compared to baseline, pre injection body temperatures. All data are represented as mean ± SEM (n=6 per group). *p< 0.05

4.3.2 Ablation of *Nucb2* protects against endotoxemic shock and LPS-induced splenic inflammation

Without an LPS challenge, all studied proinflammatory cytokines are relatively non-detectable in splenocytes from young 14-16 week old, chow fed mice. As expected, LPS injection initiated a typical acute inflammatory response in mice. Expression levels of different proinflammatory cytokines significantly increased 4 hrs after the LPS treatment. There was a fivefold increase in TNF-α ($p=0.0006$) and IL-1β ($p<0.0001$) in the spleen of wild type treated mice versus DPBS sham control mice. LPS administration also increased IL-6 levels over 300 fold ($p<0.0001$). Furthermore, expression of macrophage inflammatory chemotactic cytokines was significantly upregulated in LPS treated mice. MIP-1α expression levels increased 93 fold ($p<0.0001$) and CCL4 expression 130 fold ($p=0.0005$) in LPS treated mice.

Interestingly, LPS also increased the expression levels of these cytokines in the *Nucb2* -/- mice, however to a lesser extent as the wild type treated mice. In all the cytokines examined, *Nucb2* -/- mice had nearly half the expression of inflammatory cytokines. These data suggest the *Nucb2* deficiency significantly hinders inflammation. *Nucb2-/-* mice had 70% the level of IL-1 β ($p=0.01$) and 48% the levels of TNF-α ($p=0.01$) when compared to WT treated mice. Furthermore, *Nucb2-/-* mice had 60% the level of MIP-1α ($p=0.008$) and 42% the levels of CCL4 ($p=0.02$) when compared to WT treated mice.

Remarkably, LPS alone was able to decrease *Nucb2* expression levels in the spleen. Wild type mice treated with LPS had significantly less *Nucb2* expression. *Nucb2* levels in the wild type mice were 43% ($p=0.0005$) of those of untreated sterile mice.

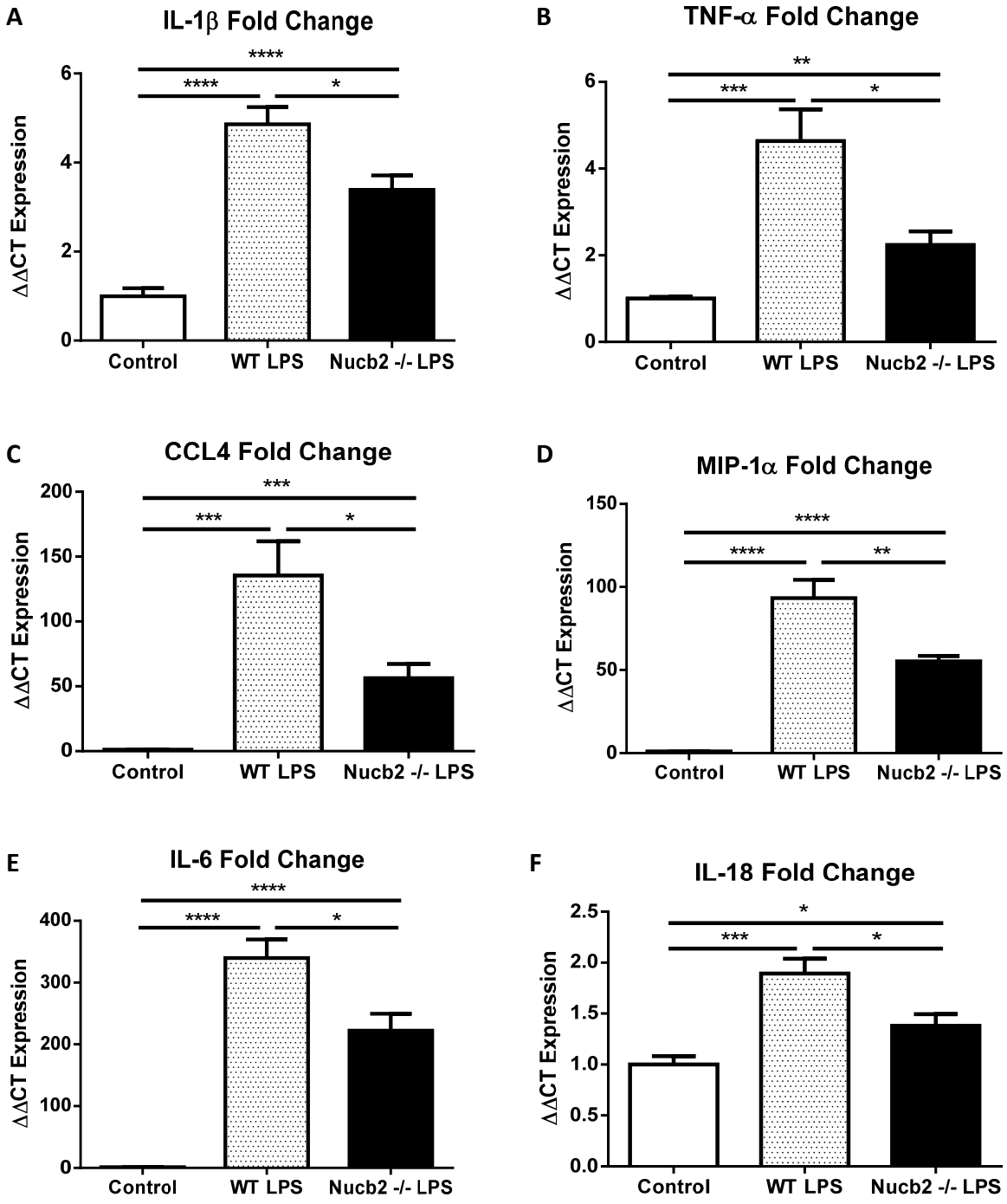

Figure 4.4: *Nucb2* ablation protects against exaggerated LPS-induced proinflammatory cytokine expression in spleen

Relative cytokine expression levels in splenocytes from sham- or LPS-treated wild type mice and Nucb2 -/- mice. (A) IL-1β relative gene expression levels (B) TNF-α relative gene expression levels (C) CCL4 relative gene expression levels (D) MIP-1α relative gene expression levels (E) IL-6 relative gene expression levels and (F) IL-18 relative gene expression levels. All data are fold change from control sham injected wild type mice and are represented as mean ± SEM (n=6 per group). *p< 0.05

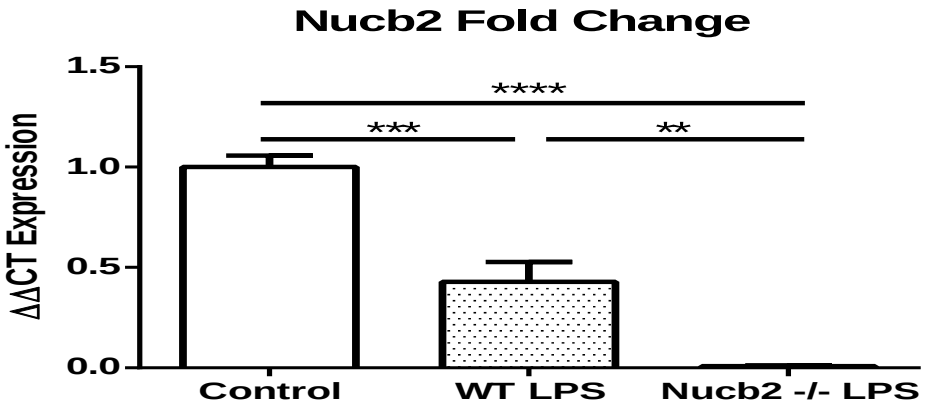

Figure 4.5: LPS inhibits *Nucb2* gene expression in spleen

Relative *Nucb2* expression levels in splenocytes from sham or LPS treated wild type mice and Nucb2 -/- mice. Data are represented as fold change from control sham injected wild type mice and are represented as mean ± SEM (n=6 per group). *p< 0.05

These data demonstrate that ablation of *Nucb2* caused a reduction in proinflammatory cytokine gene expression when animals were challenged with LPS. Very interestingly, challenging wild type mice with LPS inhibits *Nucb2* expression levels in spleen. Together, the results suggest that *Nucb2* is important in regulating inflammatory cytokine production.

4.3.3 Ablation of *Nucb2* protects against endotoxemic shock and LPS-induced systemic circulating inflammation

We next investigated whether the stimulation of the innate immune system by LPS is associated with increased levels of circulating inflammatory cytokines. In concordance with the increases seen in splenocyte cytokine gene expression, LPS challenge significantly increased serum IL-1β (WT sham vs WT LPS: 322 ± 117 pg/ml and 4323 ± 501 pg/ml, respectively, p<0.0001) and TNF-α levels (WT sham vs WT LPS injection: 276 ± 149 pg/ml and 1249 ± 144 pg/ml, respectively p=0.0008). Similarly to splenocyte cytokine gene expressions, *Nucb2-/-* mice challenged with LPS also had an attenuation of IL-1β (WT LPS vs *Nucb2-/-* LPS: 4323 ± 501 pg/ml and 2646 ± 216 pg/ml, respectively, p=0.02) and TNF-α (WT LPS vs *Nucb2-/-* LPS: 1249 ± 144 pg/ml and 823 ± 105 pg/ml, respectively, p=0.04) levels in circulation when compared to wild type LPS challenged mice (Figure 4.6). Loss of Nucb2 had no impact on LPS-induced serum levels of IFN- γ, IL-10 and IL-17 (Figure 4.6).

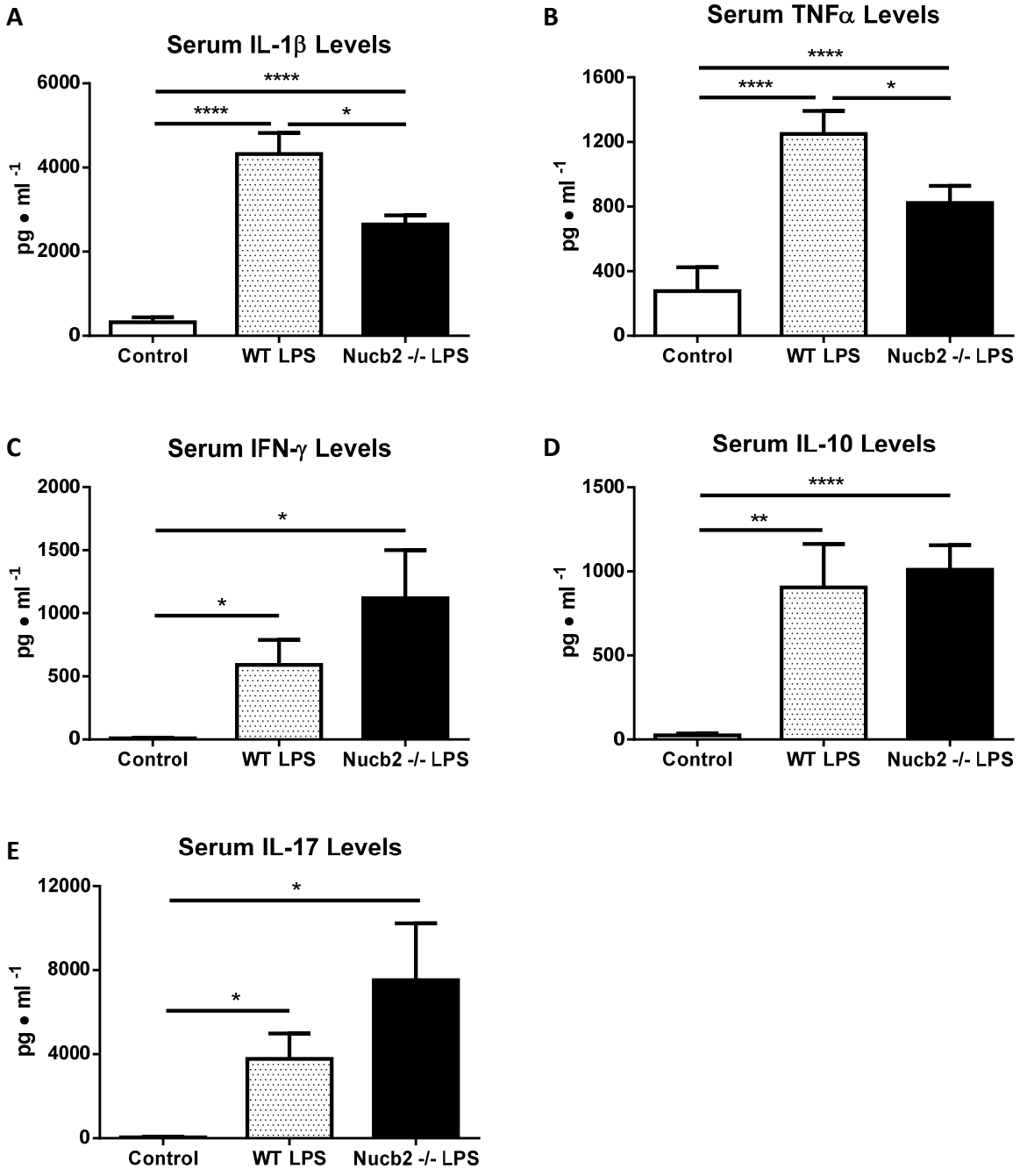

Figure 4.6: *Nucb2* ablation protects against exaggerated circulating LPS-induced proinflammatory cytokine levels

Relative protein levels in circulation in serum from sham and LPS treated wild type and Nucb2 -/- mice. (A) Serum IL-1β levels (B) Serum TNF-α levels (C) Serum IFN- γ levels (D) Serum IL-10 levels and (E) Serum IL-17 levels. All data are represented as pg/ml with mean ± SEM (n=6 per group). *p< 0.05

These data show that in models of LPS-induced endotoxemia and development of acute inflammation, loss of *Nucb2* lowers innate immune activation as reflected by reduction in proinflammatory cytokines. Ablation of *Nucb2* also decreases circulating levels of proinflammatory cytokines IL-1β and TNF-α.

4.4 Discussion

From prior data in metabolic inflammation, *NucB2* deficiency in macrophages increases inflammation in response to HFD-induced obesity. These data clearly implicated *Nucb2* as an important regulator of innate immune system in metabolic regulation. Now we are testing pathogen specific response in a model of LPS induced inflammation where inflammatory response is several log higher than obesity-induced inflammation.

We show here that ablation of *Nucb2* selectively inhibits proinflammatory cytokine production *in vivo* during LPS-stimulated endotoxic response. TNF-α has been shown to play a vital role in the pathophysiology of endotoxic shock. Using mouse models of LPS-induced acute shock, we tested the effects of *Nucb2* ablation on the production of these proinflammatory cytokines. Our findings indicate that LPS-induced endotoxemia results in similar decreases in body temperature as well as blood glucose levels in wild type and *Nucb2-/-* mice. More intriguingly, absence of *Nucb2* inhibited the production of TNF-α and IL-1β, and modified the production of other cytokines produced by monocytes in both the spleen and in circulation.

We found significant increases in proinflammatory cytokine production in the spleen. The spleen is a secondary lymphoid organ where there are abundant immune cells responsible for activation and proliferation of adaptive immunity and clonal expansion for antigen specific lymphocytes. This increase in cytokines (IL-1β, TNF-α, MIP-1α, CCL4, IL-6 and IL-18) is expected in response to stimuli. Interestingly enough, *Nucb2-/-* mice have lower proinflammatory cytokine production compared to wild type mice. As shown earlier, it is plausible that mice lacking the *Nucb2* gene have basal increases in the level of proinflammatory cytokines and that these animals are able to mount a faster immune response. This suggests that *Nucb2-/-* mice are somewhat protected against LPS induced sepsis.

In circulation, absence of the *Nucb2* gene decreased the levels of plasma TNF-α and IL-1β in response to endotoxemia when compared to LPS challenged WT mice. Although not significant, *Nucb2 -/-* mice trended towards having the ability to maintain core body temperature, a main readout for LPS induced endotoxic shock. It is important to point out that the body temperature change was only present 4 hours after LPS injection. Also, different doses of LPS might induce other pathways independent of TNF-α and IL1-β in the development of shock in this model.

Interestingly, injection of TNF-α has been shown to attenuate fever and sepsis associated with LPS endotoxemic response in rats. This was also shown in studies where antiserum against TNF-α enhanced LPS-induced fever [203, 204]. It is possible that the baseline elevated levels of proinflammatory cytokines (TNF- α) in *Nucb2-/-* mice protects these animals (or at least physiologically limits the response) against exaggerated levels of inflammation during short term endotoxemia. Moreover, others have shown that dexamethasone (Dexa), a synthetic glucocorticoid, helps alleviate inflammation, allergic symptoms, and protects against endotoxemic shock [205]. Consequently, Dexa has been widely used in treatment of sepsis in last few decades. Dexa also inhibits LPS-induced TNF-α by reducing TNF receptor affinity. Thus, Dexa counteracts the cytotoxic effect of TNF-α induced sepsis [205]. It is plausible that *Nucb2,* downstream or similarly to Dexa, may function as an endotoxin shock resistance protein in an LPS-induced endotoxemia septic shock model. Very intriguingly, independent studies show that dexamethasone significantly increases intracellular nesfatin-1 levels [134].

It is necessary to carry out longer term studies to validate and verify whether the *Nucb2-/-* mice are indeed able to resist, or at least contain, increases in inflammation during endotoxic sepsis. We therefore propose to investigate the physiologic responses to different doses of LPS. Similarly, we can investigate whether higher doses of LPS cause death in a *Nucb2* dose related effect. This would be necessary as people only succumb to LPS by an exaggerated immune/inflammatory response and not a bacterial or viral burden. We hypothesize that *Nucb2-/-* mice will be able to resist septic shock, or at least be able to limit the magnitude of response.

Chapter 5: Discussion and Future Directions

Overall and as presented in Chapter 2, 3 and 4 of this document, our data describe for the first time the important role of *Nucb2* in modulating inflammatory responses and immune cell activation states in the context of obesity and insulin resistance. Based on our results, previous data on the identity of nesfatin-1 as a secreted peptide derived from *Nucb2* is suspect. We find that the commercially available antibodies and assays to be non-specific. It is conceivable that these antibodies react with Calnuc, a highly homologous calcium-binding protein. Contrary to our initial hypothesis, *Nucb2* (or downstream nesfatin-1) is not a satiety factor and in fact does not impact feeding behaviors. As a consequence, *Nucb2* does not affect body weight or adiposity as evidenced by similar body weight and body composition in WT and *Nucb2* $^{-/-}$ mice, regardless of the composition of the diet. However, our studies show that *Nucb2* is an important gene for the maintenance of obesity induced insulin sensitivity. This effect on insulin sensitivity is however, secondary to *Nucb2's* down-regulation in proinflammatory macrophages and its impact on the production cytokines in obese adipose tissue.

In chapter 2, we showed that knocking out the *Nucb2* gene significantly impairs glucose disposal rates and therefore insulin sensitivity but only under high fat diet conditions, without affecting body weight and body fat mass. This was reflected by an almost 50% decrease in the glucose infusion rate (21.29 ± 2.09 and 11.01 ± 0.94 $mg*kg^{-1}*min^{-1}$, wild type and Nucb2 -/- respectively) necessary to maintain euglycemia in *Nucb2-/-* mice during a euglycemic hyperinsulinemic clamp, the gold standard to measure insulin sensitivity in different tissues.

Chapter 3 expands on the data presented in Chapter 2. Due to high expression of *Nucb2* in immune cells, we then set out to determine if *Nucb2* was involved in different facets of the immune system. Interestingly, *Nucb2* directly down-regulates cytokine production in Bone Marrow Derived Macrophages (BMDMs) via the NFκB cascade. First, we found that the presence or absence of *Nucb2* in BMDMs primarily impact the proinflammatory classically activated M1 polarized macrophages. This effect is translated in significantly increased production of proinflammatory cytokines in macrophages lacking *Nucb2* as measured by RT-PCR and western blot analyses. This intrinsic effect was evidenced both using *in vitro* and *in vivo* models. Furthermore, we then determined that the effects on proinflammatory cytokines production were completely attenuated when the transcriptional activity of NFκB was blocked. Together, these experiments demonstrate that *Nucb2* is required for regulation of macrophage activation, metabolic inflammation and development of the metabolic syndrome, and that *Nucb2* is working through the NFκB pathway.

Nucb2 is therefore an intrinsic regulator of the inflammatory cytokine production cascade. To further elucidate the mechanisms by which *Nucb2* impact glucose homeostasis, we

generated both adipocyte specific and myeloid specific *Nucb2* knockout mice. Our findings proved that the deleterious effects on insulin resistance is driven mostly by the lack of *Nucb2* in myeloid cells, and not in adipocytes. This is seen by impairments of insulin tolerance and glucose tolerance tests in myeloid specific, LysM^{cre} *Nucb2-/-* mice.

To follow-up these findings, we investigated functional differences in adipose tissue isolated macrophages to determine the cause of the metabolic dysfunction associated with the deletion of *Nucb2-/-*. We therefore performed genome-wide comparisons of the transcriptomes of adipocyte derived macrophages from wild type and *Nucb2-/-* mice. Using an RNA-sequencing approach, we determined that *Nucb2* is important in regulating several functional immune pathways, most being involved in increasing M1-like phenotype and decreasing M2-like phenotypes. For example, one of the most up-regulated pathways in macrophages from *Nucb2-/-* mice is that of the glycolysis pathway. M1-proinflammatory macrophages use mainly glycolysis as energy whereas M2-like macrophages utilize mainly oxidative phosphorylation. Others include increased activity of TNF pathways and tryptophan metabolism. Another very interesting difference was that involving the type I interferon response genes. It is not known, however, if type I interferons affect glucose disposal and/or insulin resistance.

Our RNA-sequencing in isolated macrophages from myeloid specific LysM^{cre} *Nucb2-/-* mice elucidated similar transcriptome differences from our isolated macrophages from global *Nucb2-/-*. Many of the upregulated pathways are involved in proinflammatory M1-like macrophage, whereas down regulated pathways are more anti-inflammatory. Due to the immune systems highly regulated states by both feed forward and feedback loops, future studies are necessary to determine where *Nucb2* is acting on each of these pathways. Are these pathways up and down regulated as a result of *Nucb2* directly or is it down stream to increased inflammatory state through NFκB pathways.

Overall, the studies presented in Chapter 3 highlight a novel mediator of immune-metabolic cross talk in the context of obesity-induced insulin resistance. *Nucb2* is an important regulator of the inflammatory cascade and is directly regulated by diet/obesity in leukocytes. High fat diet and/or obesity increase *Nucb2* levels in macrophages and T cells. In the absence of *Nucb2,* obesity induced proinflammatory macrophages are unable to downregulate inflammatory cytokines, leading to increased proinflammatory expression. This chronic, low-grade inflammation, in turn, affects whole body glucose homeostasis and triggers insulin resistance.

In chapter 4, we further show that *Nucb2* is also an important mediator of an inflammatory immunologic response to endotoxin. We show that *Nucb2-/-* mice do not have an exaggerated inflammatory response as seen in wild type mice during endotoxic shock. This is seen by decreased expression of proinflammatory cytokines in spleen as well as in the systemic

circulation by decreased IL-1β and TNF-α in *Nucb2-/-* mice. It is possible that increased levels of TNF- α and IL-1β in basal *Nucb2-/-* macrophages serve to first trigger a quick response to LPS and then contain the overall inflammatory cytokine production.

Future directions

The collected data are very convincing about the role of *Nucb2* as a modulator of inflammation and consequently insulin sensitivity. However, further studies are required for more mechanistic insights. For example, due to the pleiotropic nature and ubiquitous expression of *Nucb2,* it is critical to determine how expression is regulated and how the downstream peptides, nesfatin-1, nesfatin-2 and nesfatin-3, are also regulated. Also, there is no current knowledge on whether *Nucb2* functions through a receptor and if so what is the receptor(s). Also, it is important to distinguish between nesfatin-1, -2 and -3 and whether these different peptides have similar or dissimilar functions. It is possible that these peptides may be regulated differently by *Nucb2* and may therefore act in opposing ways or in synchrony in a physiological feedback loops way. Further studies will be done by treating *Nucb2-/-* mice with recombinant proteins of either nesfatin-1, -2 or -3 alone or in conjunction with one another.

Moreover, our data demonstrates the *Nucb2* acts through the NFκB pathway, but does not indicate through which specific pathway. Does *Nucb2* work by decreasing IkB kinase (IKK) phosphorylation thus leading to a decrease in IkB phosphorylation, and NFκB activation? Alternatively, does *Nucb2* work by a mechanism more upstream of IkB kinase? Furthermore, it is conceivable that *Nucb2* inhibits NFκB through a non-canonical IKK independent NFκB pathway. For example, *Nucb2* may directly inhibit the transcription of the subunits of NFκB, i.e. RelA or p50. Such a mechanism is possible since the *Nucb2* gene encodes for proteins with DNA binding motifs and since we see a decrease in total NFκB protein expression by western blot. To unequivocally test where in the NFκB pathway *Nucb2* exerts its effects, one can envisage to use known NFκB inhibitors that work at different steps of the pathway.

Our RNA-sequencing data show that global ablation of *Nucb2* affects type I interferon response genes in macrophages. Type I interferon is secreted by many cell types and stimulates macrophages and NK cells to elicit an anti-viral response. Interestingly, plasmacytoid (pDC) dendritic cells are responsible for production of large amounts of IFN-α and IFN-β during anti-viral response. Furthermore, according to Immgen database, pDCs have the highest expression levels of *Nucb2.* Together, these observations suggest to conduct studies designed to determine whether the ISG response seen in our macrophage RNA-sequencing is the result of the ablation of *Nucb2* in pDCs? To do this, pDCs from wild type mice and *Nucb2-/-* mice can be grown in culture and stimulated with type I interferons. Subsequently, gene expression can be examined to determine if there are differences between them. Finally, mice can be challenged with IFN-α and IFN-β to determine if *Nucb2* is required for a proper anti-viral response.

Interestingly, some studies suggest that interferon directly affects glucose tolerance and insulin sensitivity [206, 207]. It would therefore be interesting to determine if type I interferon response is involved insulin signaling and thus disrupts glucose metabolism. To determine this, we propose to stimulate glucose uptake by insulin treatment of cultured hepatocytes in presence or absence of IFN-α and/or IFN-β. Once the cells are collected, one can measure the levels of phosphorylation of the different proteins involved in insulin signaling. Such proteins would be IRS-1, AKT, MAPK and their respective phosphorylated states. Furthermore, as a supplemental model, one could use a mouse embryonic fibroblast cell line in a similar fashion. If such investigations indicate a role for type I interferon in insulin sensitivity, it will be necessary to repeat these studies in an *in vivo* model. If in vitro and in vivo studies yield similar results, this would bring even a stronger support (and mechanisms) for our observations of the impact of *Nucb2* on insulin sensitivity.

Finally, immune responses differ drastically depending on the type of infection and duration. Which other pathways of immune responses are affected by *Nucb2*? It is first imperative to repeat the LPS studies with a longer kinetics of the inflammatory response to determine if *Nucb2-/-* mice are able to better cope with endotoxemic shock. Our initial studies suggest that mice lacking *Nucb2* are able to maintain lower levels of pro-inflammatory cytokines compared to wild type mice in response endotoxemia, however this was a short term response model. It is not clear whether these mice are able to resist LPS induced shock better or if they fail to mount a proper immune response. Longer term studies will elucidate potential differences with even death as the endpoint. Furthermore, different infectious diseases and immune stimuli trigger different immune responses. It would therefore be interesting to challenge wild type and *Nucb2-/-* mice with different infectious models (for example lymphocytic choriomeningitis (LCMV) to determine if different immune responses are being regulated differently by *Nucb2.* Of interest, immunosuppressive therapies have been shown to be effective in halting many of the inflammatory diseases both in humans and in laboratory animals. It is possible that targeting the activity of *Nucb2,* or nesfatin-1, -2 or -3, be of interest for pharmacologically halting septic shock.

<u>In summary we propose the following studies</u>:

- Treatment of Bone Marrow derived macrophages with nesfatin-1, -2 and -3 alone or in combinations to determine which of the downstream *Nucb2* peptides is responsible for the modulation of pro-inflammatory cytokine production. Depending on the results, it will be important to test whether treatment of live mice (*Nucb2-/-* and wild type) with nesfatin-1, -2 and -3 affects glucose uptake and insulin sensitivity.

- Treatment of Bone Marrow Derived Macrophages from wild type mice and *Nucb2-/-* with NFκB inhibitors to test their effects on different proteins in the NFκB pathways. We can

further elucidate the mechanisms by which *Nucb2* is affecting activity of NFκB induced pro-inflammatory states in macrophages.

- Due to the difference in interferon response genes in our *Nucb2-/-* macrophages and the fact that *Nucb2* has extremely high expression in plasmacytoid dendritic cells, I propose to use cultured plasmacytoid dendritic cells from wild type and *Nucb2-/-* mice to determine if *Nucb2* is necessary for interferon response. This would further reveal potential mechanisms underlying our phenotype.

-Treatment of insulin stimulated hepatocytes with IFN-α and/or IFN-β to determine if these interferons are directly involved in insulin signaling. Depending on the obtained results, undertake treatment of wild type and *Nucb2-/-* mice with IFN-α and/or IFN-β to determine if such treatment(s) improve the insulin resistance phenotype.

-Challenge the *Nucb2-/-* mice with longer term LPS infection to determine if mice are protected from LPS induced septic shock. Furthermore, it will be necessary to use different infectious models to further elucidate *Nucb2's* role in diverse immunologic responses.

-Challenge the *Nucb2-/-* mice with different infectious models (for example lymphocytic choriomeningitis (LCMV) and *Escherichia coli* (E.coli) to determine if different immune responses are being regulated differently by *Nucb2.* E.coli would be important to determining if phagocytic pathways are modulated by *Nucb2.*

-Determine *nesfatin-1, -2 and -3* binding sites which could help identify functional elements of this protein-DNA interactions. Using either ChIP on chip or ChIP-sequencing to map global binding sites precisely. This would further elucidate if *Nucb2* proteins interact to DNA and regulate gene expression of different genes.

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Appendix

Glucose Metabolism

In the 19th century, Claude Bernard hypothesized a need for the body to maintain a stable internal environment, the *milieu interieur*, which would allow biological processes to proceed despite variations in the external environment. Bernard's concept was further developed and promoted by Walter Cannon, who coined the term "homeostasis" in describing how key physiological variables are maintained within a predefined range by feedback mechanisms [208]. Indeed, most physiological processes can only operate under a narrow range of conditions and are maintained by specialized homeostatic mechanisms in the face of variations in the environment. The body is then able to adjust in response to changes in functional demands and biological priorities. Deviations in cellular homeostasis engage some type of stress responses, which is necessary to restore homeostasis. For example, glucose metabolism can be derailed, leading to dyslipidemia, diabetes, and obesity, while amino acid metabolism seems resistant to homeostatic dysregulation.

Glycolysis is the pathway by which one glucose molecule is degraded into two pyruvate molecules in many intermediate steps through enzymatic activity. The chemical reactions begin when a glucose molecule is phosphorylated to produce glucose-6-phosphate by the enzyme hexokinase. This reaction consumes one ATP molecule, which is converted to ADP. Glucose-6-phosphate is then altered by phosphoglucose isomerase to form fructose-6-phosphate. Subsequently, fructose-6-phosphate is phosphorylated by phosphofructokinase to form fructose-1, 6-bisphosphate in a reaction that requires the hydrolysis of one molecule of ATP to ADP. At this point in the glycolysis pathway, energy in the form of 2 ATP molecules has been expended. The six-carbon fructose-1,6-bisphosphate molecule is then broken into two three-carbon molecules: glyceraldehyde-3-phosphate and dihydroxyacetone phosphate by an enzyme dehydrogenase. An enzyme can interconvert these two three-carbon sugars. Next, NAD+ is reduced to produce NADH as the each glyceraldehyde-3-phosphate molecule is converted via a kinase to 1,3-bisphosphoglycerate in a reaction requiring inorganic phosphate (Pi). When 1,3-bisphosphoglycerate is converted to 3-phosphoglycerate, substrate-level phosphorylation occurs and ATP is again produced from ADP. Then, 3-phosphoglycerate undergoes two reactions to yield phosphoenolpyruvate. Next, phosphoenolpyruvate uses pyruvate kinase to convert to pyruvate, which is the final product of glycolysis. During this reaction, substrate-level phosphorylation occurs and a phosphate is transferred to ADP to form ATP (Figure).

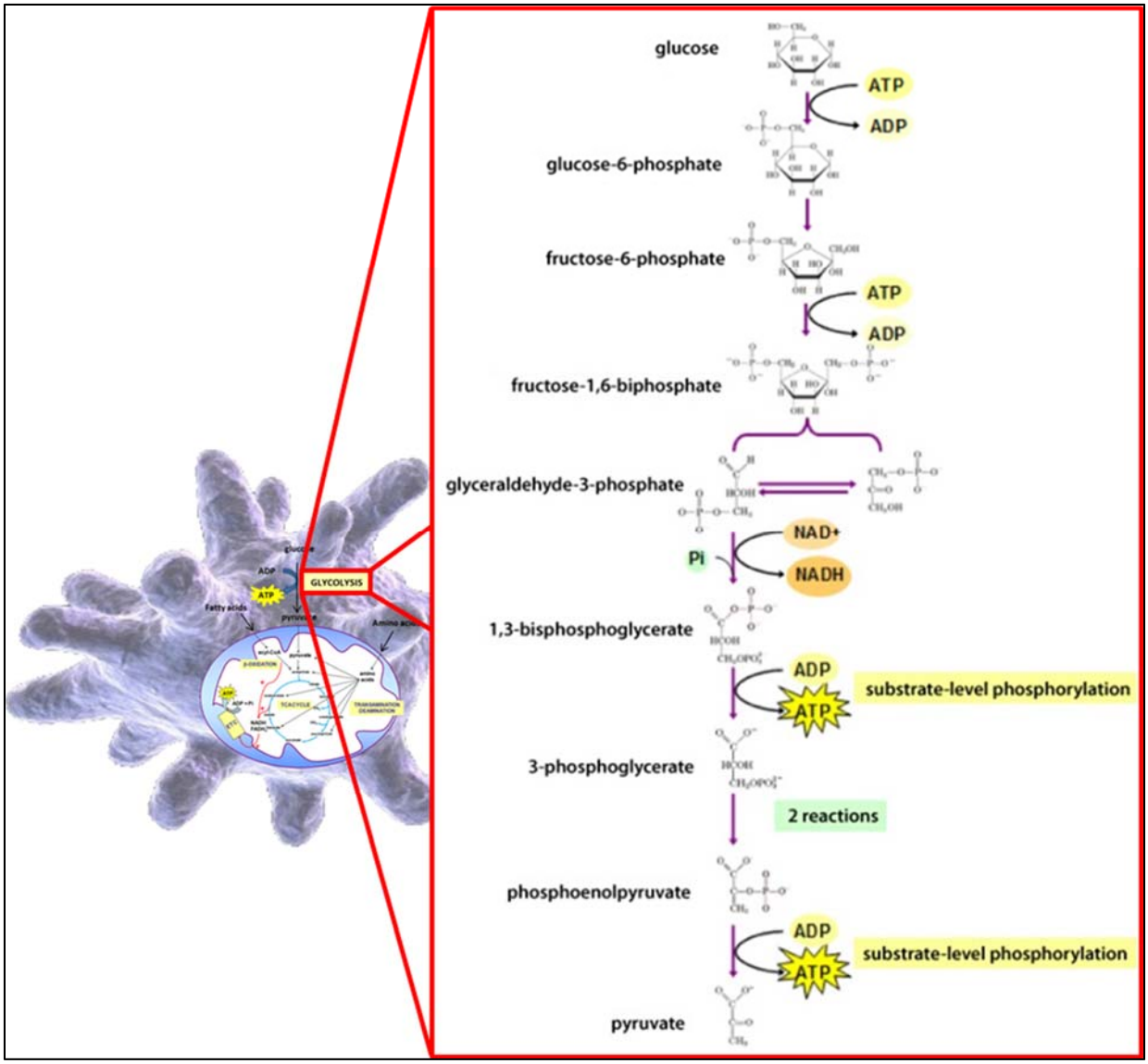

Figure: Overview of Glycolysis

The glycolysis pathway is shown in a linear diagram starting at the top with glucose and ending at the bottom with pyruvate including the intermediates and products throughout.[24]

Lipid Metabolism and Fatty Acid Oxidation

Lipids are physiologically crucial for many biological functions including, but not limited to, energy storage, signaling and the maintenance of phospholipid membranes. Lipids are either absorbed from the food we ingest or synthesized by the liver. They are hydrophobic or amphipathic, mostly insoluble in blood, and require transport within hydrophilic structures called lipoproteins. These spherical structures possess surface proteins (apoproteins) that are cofactors and ligands for lipid-processing enzymes.

The term lipid can be misguiding as it is sometimes used synonymously with fat. Fat, however, is a subgroup of lipids called triglycerides, while other lipids include cholesterol, phospholipids and fatty acids. The triglycerides constitute an important part of the lipids that circulate in the blood, and they are the main constituent of body fat in humans and animals. The triglycerides are esters derived from glycerol and three fatty acids, and their primary function is to store energy in adipocytes and muscle cells. Cholesterol, on the other hand, is a ubiquitous constituent of cell membranes, steroids, signaling molecules and bile acids.

In humans, fatty acids can be formed from carbohydrates, predominantly in the liver and adipose tissue, from the glycolytic end-product pyruvate. The first step in fatty acid synthesis starts with the carboxylation of acetyl-CoA, by acetyl-CoA carboxylase, to form malonyl-CoA, which feeds into the fatty acid synthesis pathway. Acetyl-CoA carboxylase is subject to both phosphorylation and allosteric regulation and is the point of regulation in saturated straight-chain fatty acid synthesis. Dephosphorylation of acetyl-CoA carboxylase is caused by high blood plasma levels of insulin (e.g. after meals), which in turn promotes the conversion of acetyl-CoA to malonyl-CoA, and consequently the formation of fatty acids from carbohydrates. On the other hand, glucagon, which is released into the blood during hypoglycemic conditions and exercise, causes phosphorylation of acetyl-CoA carboxylase. This in turn inhibits lipogenesis, and favors fatty acid oxidation via β-oxidation.

In other words, in times of starvation or low energy, the body uses stored fatty acids as an energy source. β-oxidation is the degradation of fatty acids, and it is named as such because the oxidation reaction occurs at the second, the β, carbon of the fatty acid chain. Fatty acid molecules are broken down to acetyl-CoA esters and furthermore are transported into the mitochondrial matrix to serve as an energy source in this catabolic process. In turn, acetyl-CoA is generated in the mitochondria and used by the citric acid cycle to produce energy. β-oxidation also produces co-enzymes $FADH_2$ and NADH, which are transported to the electron transport chain.

Tricarboxylic Acid Cycle

As previously stated, oxidation of carbohydrates, proteins and fats all converge on the tricarboxylic acid (TCA) cycle. The TCA cycle is a cycle of reactions starting with acetylCoA, a product formed from decarboxylation of pyruvate, reacting with oxaloacetate to form citrate. With the help of enzymes, citrate is then converted to isocitrate, which in turn is converted to alpha-ketoglutarate and results in the release of CO_2. Furthermore, alpha-ketoglutarate is converted to succinyl-CoA, also releasing CO_2. The cycle goes on with the conversion of succinyl-CoA to succinate, which in turn is converted to fumarate and then to malate. Malate is converted to oxaloacetate, and the cycle is complete. The oxaloacetate can react with another acetyl-CoA molecule and the cycle starts over. During the TCA cycle, electrons are transferred to NAD^+ and

FADH, to form the reduced forms NADH and $FADH_2$, which transport the electrons to the electron transport chain (ETC).

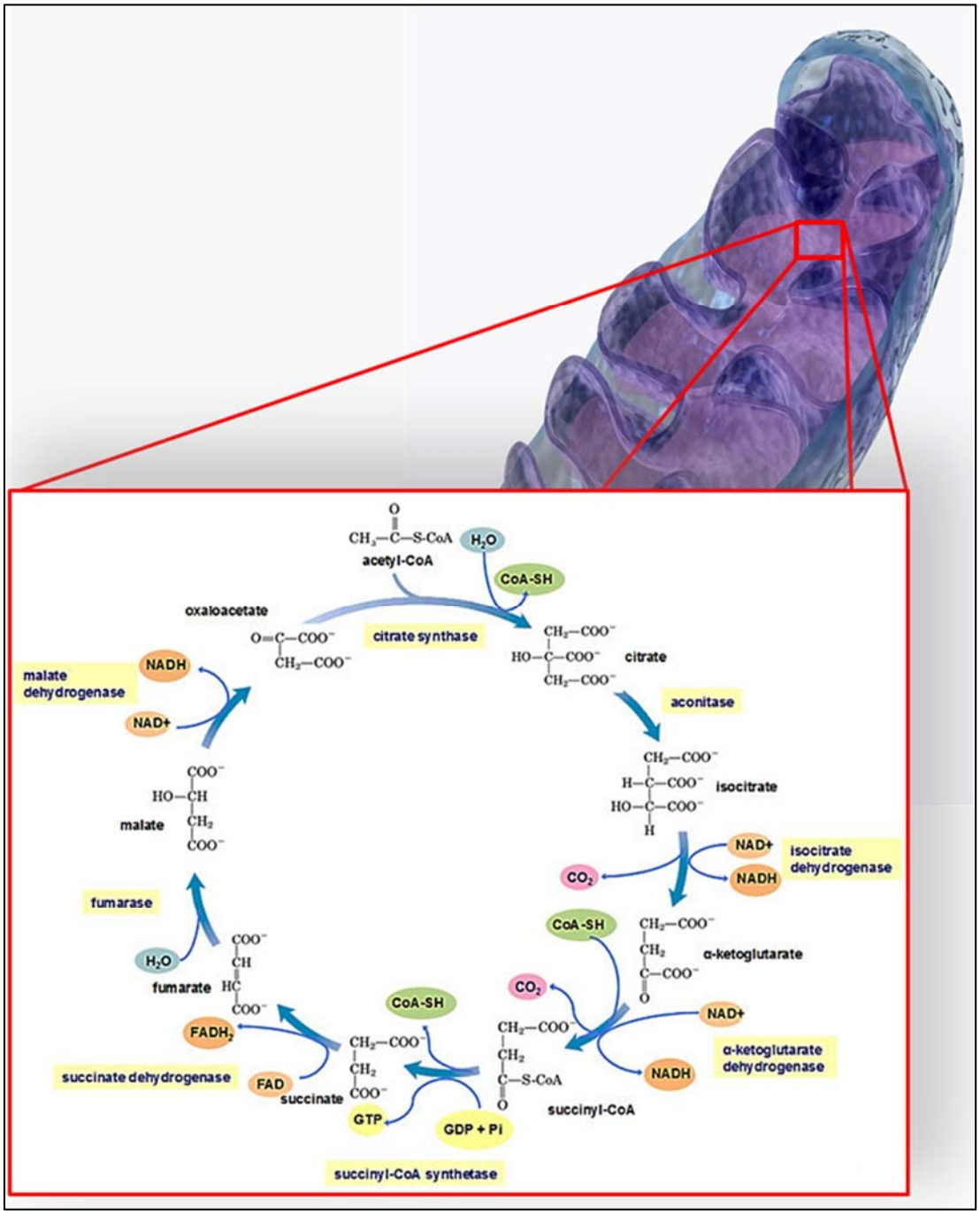

Figure: Overview of Tricarboxylic Acid Cycle

The TCA cycle is shown starting at the top with acetyl-CoA and included the intermediates and products throughout. The reactions catalyzed by the dehydrogenases that result in NAD+ and FAD reduction and the reactions catalyzed by succinyl-CoA synthetase, in which GTP synthesis occurs, are highlighted. [24]

Oxidative phosphorylation

Oxidative phosphorylation, which occurs in the electron transport chain (ETC), is the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (P_i). This is an energy-intensive process that consumes O_2 and H^+-ions to form H_2O as a bi-product. The electrons from the TCA cycle, captured by NAD^+ and FADH, provide energy to complete this process.

The collection of complexes embedded in the inner membrane of the mitochondria makes up for the ETC. Energy is produced as electrons are transferred from complex to complex along the ETC. The electrons flow spontaneously through the complexes and are coupled to the non-spontaneous ejection of H^+-ions from the mitochondrial matrix. This creates a membrane potential and a pH gradient across the mitochondrial inner membrane. The first complex of the ETC receives electrons from NADH, and the last complex transfers electrons to molecular oxygen, thus oxygen ions (O^{2-}) are formed. In the mitochondrial matrix, hydrogen ions (H^+) immediately react with the oxygen ions to form the final product, water. The strongly electronegative oxygen atoms are the final recipient of the electrons. ATP synthase, a reversible proton pump, is coupled to the ETC, and uses the energy of the proton gradient across the inner mitochondrial membrane to synthesize ATP.

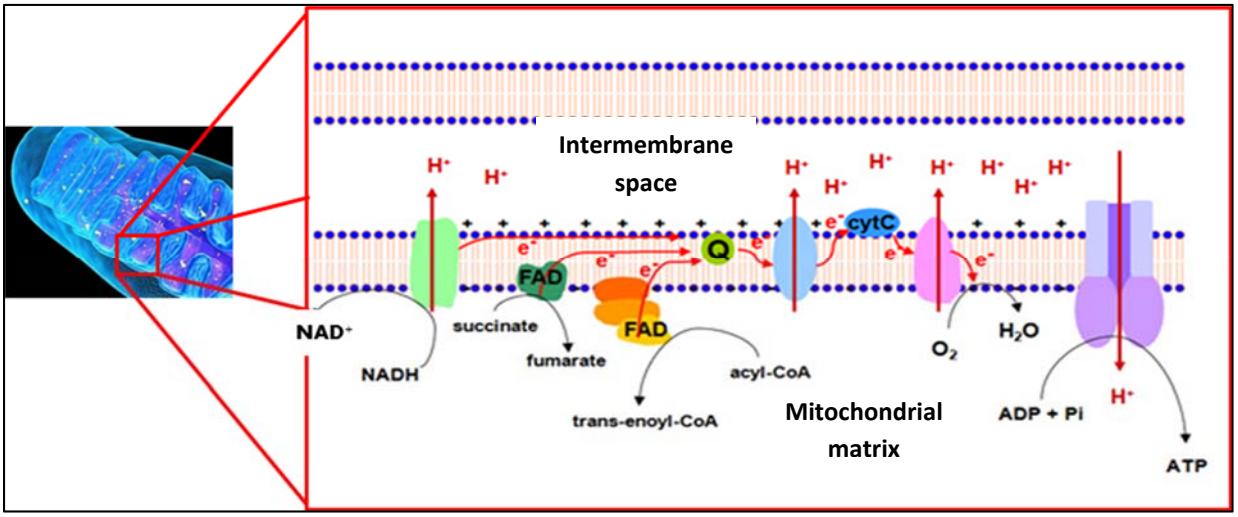

Figure: Overview of Oxidative Phosphorylation

The electron transport chain is shown in this schematic including the protein complexes that lie within the inner mitochondrial membrane. The different proteins are NADH dehydrogenase in light green; succinate dehydrogenase in dark green; the acyl-CoA dehydrogenase complex; ubiquinone in green labeled Q; cytochrome c reductase in light blue; cytochrome c in dark blue labeled cytC; cytochrome c oxidase in pink; and the ATP synthase complex in light purple. The electron flux is represented by red arrows and e-, and the proton flux is represented by red arrows and H+.[24]

Vita

Anthony Ravussin was born on December 22nd in Arizona to Jacqueline and Eric Ravussin. Anthony grew up in Arizona and attended Gold Dust Elementary School. He moved to Indiana for middle school eventually graduating from Louisiana State University Laboratory High School. Anthony began undergraduate school in the fall of 2004 at Louisiana State University, where he majored in Biological Sciences. Anthony graduated with his Bachelor of Science from Louisiana State University in spring of 2008.

In 2008, Anthony worked as an electrocardiogram technician at Our Lady of the Lake regional hospital. In 2009, he joined the laboratory of Dr. Vishwa D. Dixit at Pennington Biomedical Research Center as a research associate. In the fall of 2011, Anthony entered the graduate program at Louisiana State University in the Department of Biological Sciences. Anthony anticipates graduating with his doctor of philosophy degree in Biological Sciences in the summer of 2016 and plans to pursue a scientific career in academia.